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Fabrication of Tissue Engineering Scaffolds through Solid-state Foaming of Immiscible Polymer Blends

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Abstract

In scaffold-based tissue engineering, the fabrication process is important for producing suitable microstructures for seeded cells to grow and reformulate. In this paper, we present a new approach to scaffold fabrication by combining the solid-state foaming and the immiscible polymer blending method. The proposed approach has the advantage of being versatile and able to create a wide range of pore size and porosity. The proposed method is studied with polylactic acid (PLA) and polystyrene (PS) blends. The interconnected porous structure was created by first foaming the PLA/PS blend and then extracting the PS phase. The solid-state foaming experiments were conducted under various conditions to achieve the desired pore sizes. It is shown that the PS phase of the PLA/PS blend can be extracted much faster in the foamed samples and the pore size of the scaffolds can be easily controlled with proper gas foaming parameters. The average pore size achieved in the foaming process ranged from 20-70 μm . After PS extraction, both pore size and porosity can be further improved. For example, the pore size and porosity increased from 48 μm and 49% to 59 μm and 67%, respectively, after the PS extraction process. The fabricated porous scaffolds were used to culture human osteoblast cells. Cells grew well and gradually formed a fibrous structure. The combined solid-state foaming and immiscible polymer blending method provides a new technique for fabricating tissue engineering scaffolds.

Keywords

Immiscible polymer blends; Solid-state foaming; Tissue engineering scaffolds; Osteoblast cells; Polylactic acid; Polystyrene

1. Introduction

In scaffold-based tissue engineering, the fabrication process is important for producing suitable microstructures for seeded cells to grow and reformulate [1-4]. Many fabrication processes have been developed in the past, including fiber bonding [5], particulate leaching [6-9], three dimensional free-form fabrication [10-12], phase separation [13-17], and gas foaming [18, 19]. Recently, a new porous polymer fabrication method was developed based on immiscible polymer blends [20-22]. The approach prepares porous Poly-L-Lactic Acid

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(PLLA) from a blend of two to four immiscible polymers based on a detailed and quantitative morphology control of the blends via melt processing. A subsequent annealing step is taken to generate co-continuous phases, which lead to a fully interconnected porous PLLA matrix after extraction of the unwanted phase. Melt processing is an interesting approach to preparing polymeric materials with 100% interconnected 3-D microstructures. However, the pore size and porosity control depends on the blending ratio and the post annealing process, which can be cumbersome and challenging for large pore sizes and high porosity for tissue engineering applications [22].

Solid-state foaming is a versatile gas foaming process, where the pore size and porosity of the polymer foam can be easily controlled by adjusting the gas foaming parameters. The pore size of the solid-state foams can be fabricated from a few micrometers to several hundred micrometers, and the porosity from 10% to more than 90%. Traditionally, the polymer foams fabricated using the gas foaming method are close-celled, which prevent the in-growth of cells and the flow of nutrients [23, 24]. Many techniques were developed to improve the interconnectivity of polymer foams for tissue engineering applications. Harris *et al.* [19] developed an approach involving both the gas foaming and particulate leaching methods. Poly(D,L-lactic-co-glycolic acid) were mixed with sodium chloride (NaCl) particles and compression molded into samples of solid discs. The samples were then gas foamed and the NaCl particles subsequently removed by leaching in distilled water. This approach allowed the fabrication of polymer matrices with open-celled porous structure; however, the scaffolds exhibited non-uniform porous structure and poor mechanical strength. A process to remove the pore walls of solid state foams using ultrasound was reported by Wang *et al.* [25]. Biodegradable Poly-Lactic Acid (PLA) foams were immersed in degassed water and processed with 20 kHz ultrasound. The ultrasound created cavitation bubbles, which upon collapse generated powerful microjets that perforated the pore walls. The method was demonstrated successful for foams with pores greater than 250 μm , but was less successful with smaller pores due to the size effect of cavitation bubbles [26].

In this study we present a combined solid-state foaming and immiscible polymer blending approach to fabricating tissue engineering scaffolds with well-controlled porosity and pore size. Simple binary polymer blends were prepared with PLA and Polystyrene (PS). The composites were first foamed in the solid-state foaming process to achieve the desired pore size, and then immersed in a solvent to extract the PS phase. The advantage of the proposed process is that the pore size and porosity control can be easily achieved with the gas foaming process, while the interconnectivity of the pores is ensured with the co-continuous immiscible phases of the polymer blend. Because of the versatility of the solid-state foaming process, the combined fabrication process could provide a unique approach to creating tissue engineering scaffolds with a wide range of pore size and porosity. The fabricated porous scaffolds in this study were tested with human osteoblast cells due to their pore size and mechanical properties. It is observed that the cells gradually expanded into a 3-D fibrous structure, showing that the fabricated porous foam can be used for tissue engineering applications.

2. Experimental

2.1 Materials

PLA was obtained from NatureWorks LLC (extrusion grade, 4032D). The relative viscosity of PLA is 3.9-4.1 and the density is 1.24 g/cm^3 . The melting point is 165-173 $^{\circ}\text{C}$ and the suggested processing temperature is 210 $^{\circ}\text{C}$. The D-lactide content is 1.40 wt%, the L/D ratio is from 24:1 to 30:1, and the maximum residual lactide is 0.30%. Molecular weight (Mw) of the PLA is about 200,000 g/mol and $\text{Mw}/\text{Mn}=2$. The polydispersity index (PDI, i.e., Mw/Mn) may affect the phase separation behavior of PLA in the foaming process [13];

however, this factor was not studied in the current study due to the availability of PLA with different PDI's. PS was obtained from Dow Chemical. The molecular weight of PS is about 300,000 g/mol and Mw/Mn=2.5. The density of PS is 1.06 g/cm³. The processing temperature is 240 °C. PLA and PS were selected in this study for their compatibility in the melt processing stage. Other polymer pairs could be chosen as long as they are immiscible and have compatible processing temperatures.

The PLA and PS were blended with a 50:50 weight ratio and compression molded into thin samples. The size of the samples was 20×20 mm, and the thickness was between 0.5-0.7 mm. The density of the polymer blend was measured at 1.152 g/cm³. Medical grade CO₂ was used as the physical blowing agent in the solid-state foaming process. The CO₂ (99.95% purity) was obtained from Airgas Nor Pac, Inc. Cyclohexane (C₆H₁₂ assay by GC, corrected for water >99.0%) was obtained from Mallinckrodt Baker, Inc. as the solvent for PS extraction. Human osteoblast cells (cell line Hfob1.19, purchased from ATCC) were used in the cell culture study. Dulbecco's modified eagle medium (DMEM), Ham's F12 cell culture mediums (F12), and Fetal Bovine Serum (FBS) were used. All were purchased from Invitrogen (Carlsbad, CA).

2.2 Experimental procedure

A schematic of the fabrication process is shown in Fig. 1. Compression molded samples were foamed in a solid-state foaming process [25]. Before foaming, the sorption and desorption characteristics of the PLA/PS-CO₂ system were studied at room temperature with gas pressures at 2 and 5 MPa, respectively. During the sorption process, there samples were periodically retrieved from the pressure vessel and their weights measured with an electronic balance (resolution 10⁻³ g) in the atmospheric environment. The desorption experiments were conducted after the samples were fully saturated. The characteristics were used to determine the saturation and desorption time, which are important parameters in the foaming process.

Foaming studies were conducted using 2 MPa saturation pressure, 17.5 hour saturation time, and 10 minute desorption time. The foaming temperature was varied from 100 to 145 °C and the foaming times used were 25, 30, and 45 seconds. It is known that as the saturation pressure increases, the effective glass transition temperature (T_g) of the polymer will decrease. The foaming temperature only needs to be above the effective T_g in the solid state foaming process. It is possible to reduce the foaming temperature to room temperature if the saturation pressure is sufficiently high. Table 1 summarizes the foaming conditions used in the experiments. Three samples were foamed and characterized at each condition.

Foamed samples were selected for PS extraction in glass vials. Based on the solubility of PLA and PS, cyclohexane was selected for PS extraction. Several tests were conducted to determine the extraction time needed for the foamed samples. The samples were periodically taken out the vials, dried, and the remaining weights recorded. The percentage PS dissolved can be calculated from the remaining sample weight.

2.3 Sample characterization

FEI Siron XL 30 EDAX EDS scanning electronic microscope (SEM) was used for microstructure characterization of both the foamed and unfoamed samples in this study. The samples were prepared by freeze-fracturing in liquid nitrogen and sputter coating of a thin layer of gold. The image processing software, Image J, was used to analyze the pore size distribution. The procedure to analyze pore size distribution using Image J can be found in [27]. The porosity of the sample is defined as

$$\varepsilon = 1 - \frac{\rho_f}{\rho_s} \quad (1)$$

where ε is the porosity of the sample, and ρ_f and ρ_s are the densities of the foam and solid sample, respectively. The density of the foam can be determined using the Archimedes method [28] as

$$\rho = \frac{w_a}{w_a - w_b} \rho_w \quad (2)$$

where ρ is the foam density, w_a and w_b are the sample weights measured in air and in water, respectively, and ρ_w is density of distilled water, which is 0.9975 g/cm³ at 23°C. Since the foam density is lower than water, w_b (also called wet weight) is actually the buoyancy force measured when the sample is fully submerged in water.

2.4 Cell culture study

Porous PLA scaffolds fabricated from both foamed and unfoamed PLA/PS blends were used to culture human osteoblast cells. According to previous studies, pore sizes between 40-100 μm are suitable for osteoid in-growth [21]. All the PLA scaffolds were rinsed with purified water several times, sterilized with 70% ethanol for 30 minutes, and then subjected to ultraviolet light for 30 minutes. Sterilized samples were soaked for three days before cell seeding in a complete culture medium that contains 50% Dulbecco's modified eagle medium (DMEM) and 50% Ham's F12 culture medium, which itself contained 10% Fetal Bovine Serum FBS. The human osteoblast cells were cultured in a 25 cm² cell culture flask with a DMEM culture medium containing 10% FBS at 34 °C and 5% CO₂ (specific for this cell line based on the provided protocol). Before seeding the samples, the cells were detached from the Petri dish with 0.25% Trypsin and centrifuged at 1000 rpm for 5 minutes. The liquid at the top of the tube was discarded. Approximately 5×10^4 cells were seeded directly onto the PLA scaffolds in a 35 mm Petri dish. After one day allowing the cells to attach to the scaffolds, 2 ml complete culture medium (45% DMEM, 45% Ham's F12, 10% FBS and 0.3mg/ml G418) were added. The Petri dish was then placed into an incubator at 37 °C and 5% CO₂. The culture media were replaced every 2-3 days.

The cells were stained using a live/dead viability/cytotoxicity kit (Invitrogen) to observe them in the PLA scaffolds. The cell viability kit contained two fluorescent dyes, Calcein AM and EthD-1. Calcein AM can be well retained within live cells and show strong uniform green fluorescence (ex/em 495nm/515nm), while EthD-1 enters cells with damaged membranes and binds to the nucleic acids to produce bright red fluorescence. The scaffolds were taken out of culturing dish and an upright stereo zoom fluorescent microscope (LEICA M250 FA) was used for imaging.

3. Results and Discussion

3.1 CO₂ saturation and desorption results

The CO₂ saturation results of these PLA/PS samples are shown in Fig. 2. The equilibrium CO₂ concentrations in the polymer blends were 6.26% and 11.86% for saturation pressures at 2 and 5 MPa, respectively. The saturation results suggest that under a higher saturation pressure, the time to reach the equilibrium would be shorter. In addition, these results show that under a higher pressure the samples can absorb more CO₂, thus achieving a higher gas concentration.

Figure 3 shows the results of the desorption studies. It is seen that the gas concentration decreased at a rapid rate once it was taken out of the pressure vessel. Within twenty minutes, the samples had lost the majority of the CO₂ gained from the uptake process. For example, the gas concentration of the 5 MPa saturation samples decreased from 11.86% to about 3.96%. In contrast, the concentration decrease for the samples saturated at the lower gas pressure (2 MPa) was much slower. Even after one hour of desorption, the gas concentration only changed from 6.26% to 3.69%. This indicates that desorption time is a significant factor that will affect the foaming results. Samples foamed at different desorption times could have different pore sizes since the CO₂ concentration could be dramatically different. Therefore, desorption time was carefully controlled in this study.

3.2 The effects of foaming parameters

In general, there are three factors affecting the pore size in the solid state foaming process: CO₂ gas concentration, foaming temperature, and foaming time. The CO₂ concentration is determined by the saturation pressure and desorption time; a higher saturation pressure and a shorter desorption time result in a higher CO₂ concentration. Our previous research showed that as the CO₂ concentration increased, the pore size decreased [27]. For the samples with the same gas concentration, the foaming time and temperature would determine the pore size. The relationship between the pore size and foaming temperature and time in this study is shown in Fig. 4. Of particular interest is the strong effect of the foaming temperature. Between 100 and 125 °C, the pore size increased almost linearly from 20 to 70 μm. The foaming time also affected the pore size; however, its effect was not as significant within the 20-45 second range that was used in this study.

After the foaming temperature reached a critical point (125 °C), the bubble size decreased with the increasing foaming temperature. This was due to the fact that bubble growth in gas impregnated polymer was a result of two competing processes: the gas diffusion into the bubble and the gas diffusion out of the sample. At high foaming temperatures, the polymer matrix became softer. Hence the diffusion rate of CO₂ became much higher. As a result, more gas escapes from the sample instead of diffusing into the bubbles. Therefore, less gas was available for the bubble growth, leading to smaller bubbles. The foaming results in this study suggest that by keeping the gas concentration and foaming time constant, the foaming temperature could be used to control the pore size. The fact that the relationship between the pore size and foaming temperature is almost linear within a certain range makes it easier to control the pore size using foaming temperature.

3.3 PS extraction

Foamed PLA/PS composite samples were processed using cyclohexane and the results are shown in Table 2. The samples used for comparison were foamed at 110 °C for 30 sec. The weight loss of the foamed samples was recorded after 60 minutes of dissolution. At 180 minutes, the foam lost 4.2% of its original weight. At one day and seven days, they lost 11.0% and 28.3%, respectively. Note these results were from static dissolution at room temperature. The time can be shortened by stirring and increasing the temperature of the solution.

The microstructures of unfoamed PLA/PS blends before and after the PS extraction process are shown in Fig. 5. Figure 5(a) is the cross section of a PLA/PS sample before the extraction. It is seen that the microstructure of the sample has two distinct phases uniformly blended together: the continuous PS phase is embedded in the PLA matrix. However, no porous structure was observed. Figure 5(b) shows a PLA/PS sample after one day of cyclohexane treatment. It is seen that only a part of the PLA/PS sample became porous. This was because that it took time for cyclohexane to diffuse into the center of the sample. After

one day the solvent only reached the area below the dashed line shown in the figure. The center of the sample remained unaffected. Figure 5(c) shows the results after seven days of dissolution. Although the entire cross section of the sample was affected, there was still a large amount of residual PS left in the PLA matrix. Another observation is that the pores generated in the unfoamed PLA/PS samples reflected the geometry of the original PS phase, which was fairly small in size. Due to the PLA/PS blending process, the geometry of the PS phase exhibited an elongated shape. Therefore, the porous structure formed by PS extraction revealed channel-like pores that are 40-50 μm long, but only about 5 μm wide.

The SEM images of the foamed samples are shown in Figure 6. Figure 6(a) shows the foamed PLA/PS blend before the dissolution treatment. It can be seen that the PS phase was not foamed. Some PS clusters were located in the pores and some were found in the PLA matrix. It should be noted that the porous structure seen in Fig. 6(a) was purely because of gas foaming. Figure 6(b) shows the porous structure after seven days of PS extraction. It is seen that the PS phase was removed from the porous PLA matrix and the pores of the resulting PLA are interconnected. SEM images were also taken from the perpendicular cross section of the sample, as shown in Fig. 6(c). It is seen that the porous structure had trenches mirroring the original geometry of the PS phase. These trenches ensured the interconnectivity of the pores generated in the gas foaming process.

Comparing to Fig. 5(c), the porous structure shown in Fig. 6(c) had much larger pores, indicating that the gas foaming process was the determining factor in terms of the pore size control for the porous scaffolds. As it is well known that the pore size and porosity in the gas foaming process are easily controlled by the gas concentration, foaming temperature and foaming time, the combined gas foaming and PS extraction process can provide a convenient way to create interconnected porous structure with a wide range of pore sizes and porosity. The comparison of Figs. 5(c) and 6(c) also shows that the dissolution process of the foamed PLA/PS samples was much faster than that in the unfoamed samples. This can be seen from the fact that much less PS residuals were left in the foamed PLA/PS samples than in unfoamed PLA/PS samples after seven days of dissolution. Faster dissolution of the PS phase in the foamed samples was due to faster cyclohexane diffusion in the foamed polymer matrix.

The pore size and porosity before and after foaming and PS extraction are compared in Table 3. The pore size after foaming was $35 \pm 8.2 \mu\text{m}$ and the porosity was $49 \pm 6\%$. After seven days of PS extraction, the pore size of the extracted foam became $58 \pm 10 \mu\text{m}$ and the porosity increased to $67 \pm 4\%$. For comparison, the pore size of unfoamed PLA/PS samples after the PS extraction was only $8 \pm 3.6 \mu\text{m}$ and the porosity was only $14 \pm 7\%$. All the above values are reported with one standard deviation. This result demonstrates dramatic improvements of both the pore size (more than 7 times) and porosity (about 5 times) with the gas foaming and PS extraction process. From the percentage weight loss of the samples after PS extraction, it can be seen that the rate of PS extraction was almost doubled with the foamed samples (from $15 \pm 5\%$ to $28 \pm 7\%$). This again demonstrated the benefit of the combined gas foaming and PS extraction process for making tissue engineering scaffolds. It should also be noted that the PS extraction process used in this study is a static dissolution process at room temperature. From a practical point of view, this PS extraction process could be significantly shortened using means such as stirring and increasing the temperature of the solution.

3.4 Cell culture results

The extracted foam and unfoamed samples were used in cell culture for comparison. The cell culture results are shown in Fig. 7. The focal plane of the microscope was fixed at 200 μm beneath the scaffold surface. Images were taken at various times, including one day, one

week, and two weeks after the cell seeding. The left column of images in Fig. 7 shows the results from the unfoamed sample. The right column shows those from the foamed sample. After one day the cells were seeded, there were only a few cells that went into the scaffolds at the depth of 200 μm , as shown in Figs. 7(a) and (b). After the cells were cultured for one week, it can be seen in Figs. 7(c) and (d) that the cells had propagated and gradually spread into the scaffolds. Two weeks later more cells became visible, as can be seen in Figs. 7(e) and (f). It should be noted that the average pore size of the unfoamed PS sample was 8 μm ; however, there were larger pores in the scaffold due to the non-uniform pore size distribution. The cells grew into some of the larger pores in the unfoamed scaffold, but they were not able to proliferate to cover the entire area. Comparing to the unfoamed scaffolds, the osteoblast cells grew much better in the foamed scaffolds. The larger pores and higher porosity provided a better environment for nutrition transport and cell proliferation in the foamed scaffolds. In the unfoamed scaffolds, both the low porosity and small pore size had hampered the permeation and migration of cells and culture medium.

It has been long considered that the pore size is an important factor in tissue engineering for cell in-growth and proliferation; however, a consensus on the optimal pore size has not been reached. This may be due to the fact that there are many types of cells and each type has different cell size and growth characteristics. Sarazin et al. [21] examined published studies and provided a summary of optimum pore size requirements for several different types of cells. It was found that close to 20 μm pore size was optimal for fibroblast ingrowth as reported by some researchers, and others had narrowed it down to 5-15 μm . There are many different opinions on bone regeneration. While many suggested the optimal pore size of larger than 100 μm , e.g., 100-150 μm , 100-250 μm , 100-350 μm , and 200-400 μm , others have recommended 75-150 μm or less than 100 μm . For neovascularization, 5 μm pore size has been considered to be optimal.

Clearly there should be a trade-off on pore size selection for tissue engineering. If the pore size is too large comparing to the cell size, the cells may simply treat the pore wall as a flat surface and they may not reformulate into a 3D construct. If the pore size is too small, cells may not be able to grow into the scaffold, or they may easily plug the pores to prevent nutrient diffusion. It is conceivable that a single level of pore size may not be able to satisfy both requirements. In this case, a hybrid process may be devised to fabricate hierarchically structured tissue engineering scaffolds with large conduits for nutrient flow and small pores for cell attachment. The large conduits could be built using a 3D printing technique, while the small pores could be generated using the solid-state foaming of immiscible polymer blend technique. The advantage of the solid-state foaming of immiscible polymer blend method is that the pore size of the scaffold can be easily controlled with the solid-state foaming parameters, including the saturation pressure and foaming temperature. While a wide range of pore size can be achieved, the inter-pore connectivity is ensured with the PS phase extraction. The resolutions of 3D printing and solid-state foaming techniques are complementary; therefore, a hybrid fabrication process that takes advantage of both techniques could be used to achieve the desired hierarchically structured tissue engineering scaffolds.

4. Conclusions

This paper presented a combined solid-state foaming and immiscible polymer blending method to fabricate tissue engineering scaffolds. The processing conditions and microstructures of PLA/PS blends were investigated, followed by a cell culture study on the fabricated tissue engineering scaffolds. The results show that it is possible to control the porosity and pore size of the scaffolds in a wide range with appropriate gas foaming parameters. Extraction of the PS phase in the foamed polymer blends ensures the inter-pore

connectivity of the PLA scaffolds. In addition, foaming of the PLA/PS blends allows a much faster PS extraction rate comparing to the unfoamed samples. The results of the cell culture study show that osteoblasts could grow well in the foamed and extracted PLA/PS scaffolds. While a wide range of pore size can be achieved, the inter-pore connectivity is ensured with the PS phase extraction. The combined fabrication method provides a versatile approach to fabricating biodegradable, interconnected porous scaffolds for tissue engineering applications.

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Fig. 1. A schematic of the fabrication process

Fig. 2. CO₂ uptake in wt% of PLA/PS blend samples at room temperature for two different saturation pressures

Fig. 3. Desorption characteristics of PLA/PS blend

Fig. 4. Pores size as a function of foaming temperature and foaming time. Saturation pressure was 2 MPa, saturation time was 17.5 hours, and desorption time was 10 minutes

Fig. 5. SEM images of unfoamed PLA/PS samples showing the effects of the cyclohexane dissolution: (a) an initial PLA/PS sample, (b) after one day of PS extraction, and (c) after seven days of PS extraction. Scale bars are 20 μm

Fig. 6. The effects of dissolution treatments on a foamed PLA/PS sample (foaming temperature 110 °C, foaming time 30 sec): (a) a foamed PLA/PS sample before PS extraction, (b) after seven days of PS extraction, and (c) an image of the perpendicular cross section. Scale bars are 20 μm

Fig. 7. A comparison of cell culture results on unfoamed and foamed samples (foaming temperature 100 °C, foaming time 30 sec). PS phase has been extracted in both cases (7 days of extraction). The images were taken on a focal plane 200 μm beneath the scaffold surface at various time steps: one day after the cells were seeded ((a) and (b)), one week after seeding ((c) and (d)), and two weeks after seeding ((e) and (f)). The left column (I) shows the results from unfoamed samples. The right column (II) shows those from foamed samples. The scale bar is 200 μm each

Table 1
Parameters used in the foaming experiments

Parameters	Values
Saturation pressure (MPa)	2, 5
Saturation time (Hours)	17.5
Desorption time (Seconds)	10
Foaming temperature (°C)	100, 110, 115, 120, 125, 130, 145
Foaming time (Seconds)	20, 30, 45

Table 2
PS extraction results of foamed PLA/PS blends with Cyclohexane

Sample No.	Initial weight (g)	Duration	Final weight(g)	Percentage Dissolved
1	0.278	30 min	0.276	~0%
2	0.269	60 min	0.264	~0%
3	0.312	180 min	0.299	4.2%
4	0.326	1 day	0.290	11.0%
5	0.297	7 days	0.213	28.3%

Table 3**Pore size and porosity of the PLA/PS samples**

Samples	Dissolution time (days)	Percentage weight loss	Average pore size (µm)	Porosity
Unfoamed	unextracted	-	-	0
	extracted	15±5%	8±3.6	14±7%
Foamed	unextracted	-	35±8.2	49±6%
	extracted	28±7%	58±9.5	67±4%