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Solvent-free Fabrication of Tissue Engineering Scaffolds with Immiscible Polymer Blends

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Abstract

A completely organic solvent-free fabrication method is developed for tissue engineering scaffolds by gas foaming of immiscible polylactic acid (PLA) and sucrose blends, followed by water leaching. PLA scaffolds with above 90% porosity and 25–200 μm pore size were fabricated. The pore size and porosity was controlled with process parameters including extrusion temperature and foaming process parameters. Dynamic mechanical analysis showed that the extrusion temperature could be used to control the scaffold strength. Both unfoamed and foamed scaffolds were used to culture glioblastoma (GBM) cells M059K. The results showed that the cells grew better in the foamed PLA scaffolds. The method presented in the paper is versatile and can be used to fabricate tissue engineering scaffolds without any residual organic solvents.

Keywords

Solvent-free fabrication; tissue engineering scaffolds; pore size; porosity; GBM cells; immiscible polymer

1. Introduction

An assortment of fabrication processes for tissue engineering scaffolds have been developed in the past. These include fiber bonding [1], solvent casting and particulate leaching [2–7], three dimensional free-form fabrication [8–10], phase separation [11–17], and gas foaming [18–20]. However, many of these methods involve the use of organic solvents, which may never be fully removed even after long leaching hours. The concerns of residual solvent effects on cell growth have led to many research efforts on developing solvent-free fabrication methods for tissue engineering scaffolds [21–25].

In an early effort to avoid detrimental effects of residual organic solvents, a gas foaming method was developed [18, 26] to use a chemically inert gas, such as carbon dioxide (CO_2), as the porogen. The method was further refined by combining with particulate leaching; as a result, the inter-pore connectivity of the polymer foam was significantly improved [27, 28]. While this approach allowed the fabrication of polymer matrices with open-celled porous structure, the scaffolds suffered from non-regular porous structure and poor mechanical

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strength. The porous scaffolds had large pore sizes of nearly 400 μm , which was considered too large for many tissue engineering applications [29]. Cai *et al.* developed a phase separation and particulate leaching method and successfully created a polylactic acid (PLA)-dextran scaffold with 5–10 μm pores within the walls of 100–200 μm pores [30]. This fabrication method, however, still requires the use of organic solvents, which is a concern for long-term cell culturing.

Recently, a new scaffold fabrication method was developed based on an immiscible polymer blending approach [29, 31, 32]. Porous poly-L-lactic acid (PLLA) scaffolds from a blend of two to four immiscible polymers were fabricated via melt processing. By generating co-continuous phases among the polymers, fully interconnected 3-D microstructures can be achieved by extracting the sacrificial phase. However, the pore size and porosity control of this method relies on the blending ratio, which can be cumbersome and challenging to achieve relatively larger pore sizes and high porosity [32]. Reigner *et al.* [33] used an extrusion method to generate co-continuous poly(ϵ -caprolactone) (PCL)/polyethylene oxide (PEO) polymer blends with NaCl particulates as porogen to increase the pore size. The salt particles and PEO were then leached with water. With this method PCL scaffolds with porosities as high as 88% were fabricated. The porous structure obtained consisted of large pores (~200–300 μm) generated by salt leaching and smaller pores (~5 μm) generated by PEO leaching. However, the big pores of the PCL scaffolds were far apart and only connected through the 5 μm pores. This scaffold configuration could hamper cell spreading from one pore to another and limit the nutrient diffusion deep inside the porous scaffold. Furthermore, the weak mechanical property of PCL prevents it for being used in applications where a certain load bearing capability is required. Zhou *et al.* [34] developed a combined immiscible polymer blending and solid-state foaming method to fabricate PLA scaffolds. PLA and polystyrene (PS) were blended in a melt process. By extracting the PS phase, PLA scaffolds were achieved with pores of about 60 μm in diameter. However, organic solvent Cyclohexane was used in the PS extraction process. Although only used in the leaching step, the possible residual solvent effect could remain a concern for tissue engineering.

Here we present a completely organic solvent-free approach to fabrication of PLA scaffolds with high porosity and controllable pore size. PLA and sucrose were first blended with extrusion mixing to yield a co-continuous structure. The blends were then foamed using a solid-state foaming process, followed by immersion in water to leach away the sacrificial sucrose. This approach offers the advantage of controlling the pore size (25–200 μm) and porosity (above 90%) by simple adjustment of extrusion and foaming process parameters. In this paper, we discuss the fabrication and characterization of the solvent-free PLA scaffolds, including the effects of PLA and sucrose mixing ratio, extrusion temperature, and sucrose particle size. We characterize the mechanical properties and demonstrate the biocompatibility of the fabricate tissue engineering scaffolds. Scaffolds fabricated with and without the solid-state foaming step are also compared.

2 Materials and Methods

2.1 Materials

PLA powder was obtained from Ingeo (ECORENE™ NW 40). The relative viscosity of PLA was 3.3 ± 0.1 Pa·s and the density was 1.24 g/cm^3 . The melting temperature was 150 ± 5 °C and the glass transition temperature was 60 ± 5 °C. Sucrose was purchased from a local grocery store. Two different sucrose particle sizes were used in this study. The large particle size was $650 \mu\text{m}$ and the small particle size was $20 \mu\text{m}$. The nominal melting temperature of sucrose is 186 °C. The density is 1.586 g/cm^3 .

2.2 Polymer blending and leaching

A schematic of the scaffold fabrication process is shown in Figure 1. An immiscible blend of PLA and sucrose was prepared with a twin-screw extruder (Haake MiniLab II) in a melt process. The samples were then leached in water for 24 hours. In order to determine the best processing parameters, three independent factors: mixing ratio, extrusion temperature, and particle size were considered. Table 1 summarizes the fabrication parameters used in this study.

To determine a suitable mixing ratio, six different weight ratios (w/w) between 30/70 to 55/45 were prepared with large particle sucrose. Two extrusion temperatures 170 and 175 °C were tested with each of the mixing ratios. Once the optimal mixing ratio was identified, the effect of particle size was studied with large and small particles at extrusion temperatures of 165 , 170 and 175 °C. Both PLA and sucrose were dried at 105 °C for 24 hours before extrusion to remove moisture. A total of four grams of the mixture was loaded into the extruder for each run. The extrusion condition for all the samples was one pass flush at a screw speed of 100 rpm. The samples were subsequently immersed in water for 24 hours and dried at 105 °C for two hours.

2.4 Solid-state foaming

Samples with the PLA-to-sucrose weight ratio of 35/65 and extruded at 165 and 170 °C were foamed in a glycerol bath using a solid state foaming method [34]. The samples were saturated in a high pressure vessel using CO_2 at a pressure of 2 MPa. A saturation time of 72 hours was applied in order to achieve the full saturation of CO_2 in PLA. The samples were then foamed at 50 °C for 45 seconds immediately after they were taken out of the pressure vessel. The foamed samples were cut into two pieces, one leached in 100 ml of deionized water with stirring and the other used as a control for comparison.

2.3 Sample characterization

A scanning electron microscope (SEM) (JOEL 5600) was used to characterize the microstructure of the samples both before and after water leaching. The samples were prepared by freeze-fracturing and sputter coating a thin layer of Au-Pd. Image processing software (Image J) was used to analyze the pore size distribution. As the pores were not spherical, an average of the largest and smallest Feret diameter (the greatest distance possible between any two points along the boundary of the pore) was used to represent the pore size. The densities of the sample before and after water leaching were measured with

the liquid displacement method described in ASTM D792 [35]. The porosity of the sample was defined as the ratio of the foam density to PLA bulk density.

Mechanical properties of the samples were tested with a dynamic mechanical analyzer (TA DMA Q800). Samples of 10–15 mm long, 3 mm wide, and 1 mm thick were used to measure the dynamic modulus of the material. The dynamic modulus was calculated from the storage modulus and loss modulus as follows.

$$E = \sqrt{(E')^2 + (E'')^2} \quad (1)$$

where E is the dynamic modulus, E' is the storage modulus, and E'' is the loss modulus. The sinusoidal driving force was set at the amplitude of 1 N and a frequency of 1 Hz.

All data are expressed as mean $\pm$ standard deviation (SD). The Student t-test was used to analyze the statistical significance of pairs of data. The significance was considered when $p < 0.05$. A p -value larger than 0.05 ($p > 0.05$) was taken as an indication of no significant difference.

2.5 Cell culture study

To demonstrate the biocompatibility, porous PLA scaffolds fabricated with the 35/65 PLA/sucrose blending ratio were selected for cell culture using glioblastoma multiforme (GBM) cell line M059K. GBM cells were chosen to test if the scaffolds were suitable for creating brain tumor models that are useful for *in vitro* cancer drug screening [36]. All the PLA scaffolds were rinsed with purified water, sterilized with 70% ethanol for 30 minutes and exposed to ultraviolet light for 30 minutes. Sterilized samples (10mm $\times$ 3mm $\times$ 1mm) were immersed in complete cell culture medium (DMEM with 10% FBS) for three days before cell seeding. The M059K cells were first cultured in a 25 cm² cell culture flask with the complete cell culture medium at 37°C and 5% CO₂ in an incubator. Before seeding the samples, the cells were detached from the flask with 0.25% Trypsin and centrifuged at 1000 rpm for five minutes. The cells were re-suspended with culture medium and seeded onto the PLA scaffolds in a 24-well cell culture plate. Each sample received 100 μ l cell and medium solution, which contained approximately 10⁵ cells. After one day allowing cell attachment, 1 ml complete cell culture medium was added to each sample. After another 2 days the samples were transferred to another 24-well cell culture plate to ensure that the cells were all growing in the scaffolds. Three replicates were tested for each condition. The cell culture plate was maintained in an incubator at 37 °C and 5% CO₂. The culture medium was replaced every week.

The cells were stained using a live/dead viability/cytotoxicity kit (Invitrogen) for observation. 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 495 nm/515 nm), while EthD-1 enters cells with damaged membranes and binds to the nucleic acids to produce bright red fluorescence. The cells were stained for 20 minutes and observed using a stereo zoom fluorescence microscope (LEICA M250 FA).

Cells inside the PLA scaffolds were also observed using SEM. Scaffolds after 14 days of cell culturing were cut in half and the cells were fixed with Karnovsky's fixative overnight at room temperature, followed by dehydration in 75, 90, 95% ethanol successively for 15 minutes each and finally with 100% ethanol for three times, each time with 15 minutes. The samples were then coated with carbon for imaging.

3. Results and Discussion

3.1 Effects of mixing ratio

Figure 2 shows the SEM images as well as a schematic of the morphology change in the PLA-sucrose blend with increasing amounts of PLA. When the mixing ratio of PLA and sucrose was 30/70 by weight, the microstructure of the blend consisted of a sucrose matrix with isolated PLA domains. When the PLA ratio was increased to 35/65, the two phases became co-continuous. When the PLA ratio was increased to 45/55, a PLA matrix with isolated sucrose domains resulted.

The effect of mixing ratio can also be observed from the data on weight loss after water leaching, shown in Table 2. All the samples were immersed in water for 24 hours for sucrose leaching. For samples fabricated at 170 °C, immersion for 24 hours was sufficient to leach all the sucrose when the sucrose weight ratio was above 60% (*i.e.*, volume fraction of 55%). For samples with sucrose weight ratio below 60%, the weight loss due to leaching decreased with the decreasing sucrose ratio. With decreased sucrose content, the sucrose phase became increasingly isolated in the PLA substrate, resulting in incomplete leaching. This trend was similar for samples mixed at both 170 and 175 °C; however, the overall weight loss for 175°C samples were less significant compared to the 170°C samples. This was because that at 175 °C sucrose started to decompose and become caramel, which has a lower solubility in water.

3.2 Effects of extrusion temperature and sucrose particle size

In an effort to further determine the effects of extrusion temperature and sucrose particle size, a number of samples were prepared using extrusion temperatures of 165, 170 and 175 °C with large (650 µm) and small particle (20 µm) sucrose. The PLA to sucrose mixing ratio was fixed at the 35/65 w/w ratio. These samples were not foamed using the solid-state foaming process, such that the effects of particle size could be clearly observed. Figures 3 and 4 present SEM images of the microstructures using large and small particle sucrose particles, respectively. The small particle samples yielded cross sections notably smoother than the large particle samples. Even before leaching, pores were clearly observed in the large particle samples, possibly because of the partial melting of sucrose and the poor bonding between sucrose particles and the PLA matrix. After leaching, the large and small particle samples revealed distinctive porous structures. The large particle samples had two levels of pore sizes. The large pore size level was caused by the sucrose particles that did not completely melt during mixing. The small pore size level was caused by the co-continuous structure formed by molten sucrose and PLA. When the sucrose particle size was small, the melting was generally complete, thus the porous structure was mainly caused by the co-continuous structure of PLA and sucrose. Figure 4 also shows that the mixing temperature

affected the domain size of sucrose in the PLA/sucrose blends. Mixing at 165 °C appeared to cause smaller sucrose domains; therefore, the pores formed after leaching were smaller, as compared to those at 175 °C.

The average pore sizes of the above samples at each extrusion temperature and sucrose particle size are shown in Figure 5. Regardless of the sucrose particle size, the pore size of the fabricated scaffolds reduced with a decreasing mixing temperature. As expected, the pore sizes of the small particle samples were smaller than those of the large particle samples at corresponding mixing temperatures. However, the mixing temperature seemed to have a stronger effect than the particle size. The porosities of these samples are shown in Table 3. While it varied from 50–65%, the porosity was higher at 170 °C compared to those at other extrusion temperatures. This was true for both large and small sucrose particle samples.

3.3 Mechanical properties

The dynamic modulus measurements of samples both before and after water leaching are also shown in Table 3. The mechanical property of Sample 6 was not available due to excessive brittleness of the sample. As expected, the dynamic modulus before leaching is much higher with the sucrose component intact. This can also be seen from Figure 6(a), where the dynamic modulus data were compared across different extrusion temperatures. Overall, the dynamic modulus reduced after water leaching, as the materials became porous. The extrusion temperature also had a significant effect on the mechanical property. As the extrusion temperature increased, the samples after leaching showed a reduced dynamic modulus. With similar porosities for all the samples, this decrease in modulus is attributed to the increase of pore size, as it is generally accepted that the porous material with a large pore size would have a lower mechanical strength, keeping the porosity constant. This pore size effect, however, does not apply to samples prepared with different sucrose particle sizes. As seen in Figure 6(b), the dynamic modulus was higher when large particle sucrose was used, even though the pore size was larger (88 μm as compared to 48 μm for small particle samples). This was because of the morphological difference between the two types of scaffolds, as seen in Fig. 4. The large particle samples had large pores in a matrix of smaller pores, while the small particle samples had a relatively uniform porous structure. On average, the large particle samples had a larger pore size; however, the small pore sized matrix may have contributed to a higher dynamic modulus comparing to the small particle samples.

3.4 Effect of solid-state foaming

The effect of solid-state foaming is shown in Figure 7 with samples fabricated at 165 °C and the PLA/sucrose weight ratio of 35/65 using large particle sucrose. For the foamed sample before leaching, there were visible pores randomly distributed in the PLA matrix, which demonstrates that solid state foaming can be used to establish porous structure inside the PLA and sucrose blend. The porosity of foamed blend without water leaching was measured at 34.1%. It should be noted that sucrose is a crystalline material and cannot be foamed using the solid state foaming process. Therefore, the porosity generated by foaming was completely in the PLA matrix. Figure 7(a) shows the cross section of an unfoamed sample after 24 hours of leaching. The large pores seen on the level of tens of microns were

generated by leaching of sucrose particles that did not completely melt. Much smaller pores were observed on the walls of these large pores. This structure is similar to those obtained in [33], where co-continuous PCL and PEO polymer blends were made into tissue engineering scaffolds using a salt leaching technique. The large pores in the porous structure were only connected through the much smaller pores. The porosity of the sample was about 60%, although a higher porosity could be obtained by using larger sized particles. Figure 7(b) shows the cross section of a foamed sample after water leaching. A large pore structure formed by particulate leaching was found similar to that shown in Figure 7(a). In addition, there were many median sized pores on the level of 20–30 μm generated by the solid-state foaming process. These median sized pores were on the walls of large pores and connected them together. The porosity was 91%. Such a porous structure is more conducive to cell growth and nutrient transfer, as will be shown with the cell culture results.

3.5 Cell culture results

Both unfoamed and foamed samples after leaching were tested in cell culture studies for comparison. The PLA and sucrose blends were mixed with a weight ratio of 35/65 at an extrusion temperature of 170 °C. Fluorescence images were taken at the 7th and 14th day after cell seeding. As shown in Figure 8, cells grew well on both unfoamed and foamed samples up to 7 days. The bright green dots show individual cells. However, the cell densities on the foamed samples were much higher than those on unfoamed samples, suggesting that foamed samples provided a much better environment for cells growth. After 14 days, the cell density on the unfoamed sample was even lower than that after 7 days, suggesting that the unfoamed samples would not support long-term cell culture due to the potential problems in cell spreading and nutrient transfer. Figure 9 shows a cross section of a foamed sample after 14 days of cell culture. Cells were seen populated throughout the porous scaffold. They remained in round morphology and attached to the walls of the large pores. It is believed that the foaming process enlarged the pores in the porous scaffold, which facilitated easy transfer of nutrients and cell migration.

3.6 Discussion

Scaffold fabrication is one of the important issues in tissue engineering. A scaffold without organic solvents and with a hierarchical porous structure is critical to cell growth. The scaffold fabrication method presented in this study is a completely solvent-free approach, with the capability to control pore size and porosity easily through process settings such as the extrusion temperature and foaming parameters. By adjusting the sucrose particle size and extrusion temperature, large pores can be controlled on the level of $\sim 200 \mu\text{m}$; by controlling the foaming parameters the small pores can be achieved on the level of $\sim 25 \mu\text{m}$. Such a hierarchical porous structure allows better cell attachment and easier nutrient transport, both of which are beneficial for long-term cell culturing. It has been reported that different tissue engineering applications will require different pore sizes for scaffolds. For example, pores of 20–125 μm are suitable for skin regeneration. Bone regeneration will require many pore sizes from 75–150 μm to 200–400 μm depending on different cell types. The scaffold fabrication method developed in this study may be utilized for a wide range of tissue engineering applications because of its versatility in pore size and porosity control. Moreover, the developed fabrication process in this study employs sucrose as the sacrificial

phase, instead of sodium chloride that has been used in previous studies. As a sacrificial phase, there is possibility that the porogens are not fully leached away. Residue sodium chloride particles trapped inside the scaffold are detrimental to cells and may cause DNA breakdowns [37]. Sucrose is biocompatible. Even not fully removed, it has little chance to cause detrimental effects to cells.

4. Conclusions

A completely solvent-free fabrication method for tissue engineering scaffolds has been presented. Immiscible polymer blends of polylactic acid and sucrose were obtained using twin-screw extrusion and foamed using the solid-state foaming process. The co-continuous structure of PLA and sucrose was obtained at the 35/65 weight ration. After leaching, foam PLA scaffolds with above 90% porosity and 25–200 μm pore size could be achieved. The pore size and porosity of the PLA scaffolds can be easily controlled by adjusting the process parameters, including mixing ratio, extrusion temperature, and foaming temperature. Cell culture studies suggested that cells grew better in foamed PLA scaffolds. The developed fabrication method is versatile and can be used to avoid the residual solvent problem in tissue engineering applications.

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Figure 1.
A schematic of the fabrication process

Figure 2.

SEM images showing polymer blends with different weight ratios mixed at 170 °C. The scale bars are all 50 μ m.

Figure 3.

SEM images of large sucrose particle samples before and after water leaching: (a) and (d) at 165 °C, (b) and (e) at 170 °C, (c) and (f) at 175 °C. The scale bars are all 100 µm.

Figure 4.

SEM images of small sucrose particle samples before and after water leaching: (a) and (d) at 165 °C, (b) and (e) at 170 °C, (c) and (f) at 175 °C. The scale bars are all 100 μm .

Figure 5.

Average pore size at different extrusion temperatures. The results are presented as mean $\pm$ SD from three independent samples.

Figure 6.

Mechanical properties of PLA and sucrose blends at 35/65 w/w ratio before and after water leaching: (a) Large particle samples mixed at 165, 170 and 175 °C; (b) different particle sizes at 170 °C. The results are presented as mean±SD with 3–5 independent measurements, * $p < 0.05$.

Figure 7.

SEM images of PLA/sucrose (35/65, large particle, 165°C) samples: (a) unfoamed with water leaching; (b) foamed with water leaching.

Figure 8.

A comparison of cell culture results with unfoamed and foamed samples (35/65, large particle, 170°C): (a) Unfoamed at Day 7, (b) foamed at Day 7, (c) unfoamed at Day 14, and (d) foamed at Day 14. All samples were leached for 24 hours. The scale bars are all 2mm.

Figure 9.

An SEM image of cells inside a foamed PLA scaffold (35/65, large particle, 170°C) after 14 days of culturing. The scale bar is 50 μm.

Table 1

Parameters used in the fabrication process

Parameters	Values
PLA: Sucrose ratio (wt/wt)	30:70,35:65,40:60,45:55,50:50,55:45
Extrusion temperature (°C)	165,170,175
Sucrose particle size (µm)	20, 650
Extrusion process	One pass
Total weight per run (g)	4
Screw speed (rpm)	100

Table 2

Weight loss after 24 hours of water leaching

		Weight Loss at Different Mixing Ratios (PLA/Sucrose)							
Extrusion Temperature (°C)	w/w	30/70	35/65	40/60	45/55	50/50	55/45		
	v/v	35/65	41/59	45/55	51/49	56/44	61/39		
170		71.7%	69.9%	63.1%	49.9%	46.1%	32.6%		
175		68.0%	59.4%	55.7%	48.5%	40.8%	32.5%		

Table 3

Microstructural and mechanical properties

Sample No.	Mixing ratio (w/w)	Extrusion temperature (°C)	Sucrose Size (µm)	Porosity (%)	Pore size (µm)	Dynamic Modulus (Before leaching) (MPa)	Dynamic Modulus (After leaching) (MPa)
1	35/65	165	650	59.7%	74±21	3365±1007	2062±193
2	35/65	170	650	64.9%	88±22	3803±869	1320±509
3	35/65	175	650	59.7%	174±33	2673±1331	1057±328
4	35/65	165	20	50.4%	48±13	4611±65	1961±526
5	35/65	170	20	63.9%	81±24	2922±41	698±259
6	35/65	175	20	57.0%	135±38	-	-

Error was expressed as +/- one standard deviation