Preparation of Highly Porous Interconnected Poly(lactic acid) Scaffolds Based on a Novel Dynamic Elongational Flow Procedure

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ABSTRACT

Sodium Chloride (NaCl) is a common porogen to be used in preparing porous scaffolds in tissue engineering. However, NaCl particles are easily broken during melt blending with the matrix polymer by conventional processing techniques, such as twin extrusion, which causes uncontrollable pore size and entrapped residues after leaching in the prepared scaffolds. To address these problems, this study reported a novel scaffold fabrication method based on self-developed vane extruder, which applies global dynamic elongational flow to the polymer compounds. We found that NaCl particles not only preserved their original size, but also could retain high loading content when been blended with poly(lactic acid) (PLA) matrix using vane extruder. Based on these high porogen composites, we are able to prepare PLA porous scaffolds with porosity as high as 93% via supercritical carbon dioxide (Sc-CO₂) foaming and particulate leaching hybrid method. Detailed analyze confirmed that the NaCl porogens could be completely leached within 24 h. The biocompatibility of the scaffolds prepared was verified by culturing human mesenchymal stem cells. Therefore, the proposed approach based on vane extruder compounding has great potential to be used in tissue engineering scaffold fabrication.

KEYWORDS: Poly(lactic acid); vane extruder; dynamic elongational flow; gas foaming; particulate leaching; tissue engineering scaffold

1. INTRODUCTION

Tissue engineering scaffolds are used as temporary templates to support the regeneration of new tissue [1, 2]. An ideal scaffold should be highly porous with an interconnected, open porous structure to provide a three-dimensional (3D) substrate for cell growth and nutrition transport. Various techniques have been introduced or developed to produce porous scaffolds, such as fiber bonding [3-5], solvent casting /particulate leaching[6-8], phase separation technology[9-11], gas foaming[12-15], and solid freeform fabrication[16-18]. However, so far, there is no a single method that can produce scaffolds meet all requirements of tissue regeneration because of the complex *in vivo* environment and the diversity of tissue kinds. Therefore, hybrid methods have been attracting attentions in recent years in order to combine the advantages of different technique and overcome their disadvantages.

The great advantage of solvent casting/particulate leaching over other approaches is that it is capable of directly controlling the pore size and structure by adjusting the porogen size and concentration [6-8]. However, one drawback of this technique is that the presence of organic solvent, which is hard to completely remove from the scaffolds and the release of the residue solvent in the scaffolds will be harmful for cell growth and nearby tissues when implanted. Another drawback of this method is the difficulty in production of three-dimensional structures. To overcome these problems, melt-molding/particulate leaching was proposed to replace the solvent casting step [19, 20]. Although the organic solvent is avoided, the potential deficiency of this technique is the lack of interconnectivity between the pores at low volume fraction of porogen particles. To improve the interconnectivity between the pores, conventional immiscible polymer blending technique was adapted to produce interconnected, porous, three-dimensional scaffolds [21, 22]. The strategy consists of melt blending of two immiscible polymers to create

so called co-continuous blending morphology. One of the polymer phases is subsequently sacrificed by selective removing from the blends to form the continuous channels within the polymeric matrix. Typically, the sacrificial phases used in this technique are water soluble polymers.

To reduce the cost and improve efficiency of scaffold fabrication, and develop a more controllable approach, researchers have been trying to employ the melt blending and injection molding techniques in scaffold preparation. However, the drawback of these techniques is that the maximum porosity achieved was 50-60% which is not suitable to be used as tissue engineering scaffolds without further modification. Recently, Reignier et al. have improved the above method by combining the polymer leaching and particle leaching to obtain highly porous interconnected networks[23]. By removing the sacrificial polymer phase and porogens, the porosity of the scaffolds can be achieved to 88%. To further improve the porous structure of the scaffolds, Botchway et al. combined gas foaming with polymer leaching technique to prepare poly(ϵ -caprolactone) (PCL) scaffolds without the use of NaCl particles and harsh chemicals[24]. Turng group employed polymer/particle leaching combined with gas foaming to prepare porous PLA, PCL and thermoplastic polyurethane (TPU) scaffolds. In the studies, water soluble polymer such as poly (vinyl alcohol) (PVA), poly (ethylene oxide) (PEO) and NaCl as porogens were first blended with polymer matrix such as PLA, PCL and TPU. The prepared blends were then foamed with supercritical CO₂ or N₂ to obtain the foamed blends. After leaching the water soluble phases, porous scaffolds with multimodal porous structure and interconnected channels were obtained [25-28].

However, the most significant drawbacks in these methods is the difficulty to retain porogen size and to achieve high porogen concentration. It is well known that the polymer melts subject

to strong shear deformation in the screw extrusion process. High shear stresses especially in the melting zone can cause great attrition of particulate, which affects the pore structure of porous scaffolds consequently [23, 28]. In addition, the inorganic porogens such as NaCl have different properties from polymer melts. They are difficult to achieve good dispersion in polymer matrix under shear deformational flow. To address these problems, elongational flow field was proposed and it is widely recognized that elongational flow in polymer processing has many advantages over the conventional shear deformational field [29]. Recently, inspired by such concept, we have recently designed a novel polymer processing equipment, known as vane extruder, which applies strong elongational flow to polymer melt during processing by consecutively compression and stretching using a series continuous dynamical converging channels [30-32]. Experimental study demonstrated that the vane extruder has a much higher dispersive mixing efficiency than twin-screw extruder. Moreover, compared to shear deformation field, elongational field consumes less energy to generate the same deformation of polymer [33].

The purpose of this study is to prepare porous scaffolds using vane extruder combined with particulate leaching and Sc-CO₂ foaming techniques. Poly(lactic acid) (PLA), a popular biodegradable polymer, was chosen as the scaffold matrix material. NaCl and PVA were used as water soluble porogens. The effect of NaCl concentration on the scaffold morphology and the porosity was investigated. Human mesenchymal stem cells were used as cell model to investigate the potential of the prepared scaffolds for bone tissue engineering.

2. EXPERIMENTAL

2.1 Materials

Biodegradable PLA (2002D, $M_w = 208,000$ g/mol, 4.25% D-lactic acid) was supplied by Nature Works LLC. It has a melt flow index of 3.4 g/10min (190 °C, 2.16 Kg) and a density of 1.24 g/cm³. The PLA pellets were dried at 80 °C in vacuum oven for 4 h before use.

PVA (GM-14S) with density of 1.27 g/cm³ was provided by Japanese Synthetic Chemistry Corp. It has been 88% hydrolyzed with an average polymerization degree of 1700. The material in powder form was dried at 60 °C in vacuum oven for 8 h before use.

Edible NaCl with a specific gravity of 2.16 g/cm³ was purchased from Guangdong Province Salt Industry Group Corp., China. It was sieved to desired size ranging from 75 to 150 μ m, and dried for 8 h at 80 °C under vacuum prior to using.

Commercial purity grade CO₂ (purity grade, 99%) used as physical blowing agent was purchased from Shengtong Corp., China.

2.2 Vane Extruder Device

A novel polymer processing equipment known as vane extruder was employed in this study to prepare the PLA/PVA/NaCl blends. The structure schematic of the vane extruder is shown in Figure 1. Instead of using screws in conventional extruder, the vane extruder is comprised of several vane plasticizing and conveying units (VPCUs) along the axial direction. The stator, vanes, baffles and the rotor of VPCUs composes a closed chamber. The volume of the closed chamber changes periodically as rotor rotates during processing because of the eccentric distance from the stator to the rotor. A dynamical converging channel is obtained along the circumferentially direction. Thus, high stress and dynamic elongational flow field could be generated. The vane plasticizing units feed materials as the volume increases and discharge

materials as the volume decreases. The detailed working principle of the vane extruder has been introduced in the previous studies[30]. The vane extruder used in this study consists of 12 VPCUs. The inner diameter of the stator is 46 mm, the outer diameter of the rotor is 40 mm, and eccentric distance between the rotor and stator is 3 mm.

2.3 Preparation of Porous PLA Scaffolds

Pre-dried PLA, PVA and NaCl were dry-mixed at certain proportions and compounded via the vane extruder at a rotation speed of 100 rpm. The temperature profiles were 160 °C, 170 °C, 170 °C, 160 °C from hopper to die. For the melt blending, the PLA/PVA was kept constant at 50/50 by volume to ensure a co-continuous morphology of the polymer matrix, while the NaCl content was varied from 10% to 40% of the polymer matrix by volume. Then, the prepared PLA/PVA/NaCl blends were saturated with Sc-CO₂ in a high-pressure vessel. The vessel was heated to the saturation temperature 150 °C in 1 h. Then, the vessel was filled with Sc-CO₂ to desired saturation pressure 10 MPa. The sample was kept in equilibrium at 150 °C and 10 MPa for 1 h and subsequently cooled to the foaming temperature of 100 °C. Then, the high-pressure vessel was instantaneously depressurized to the atmospheric pressure to induce cell nucleation and growth. The sample was solidified by water-cooling system. Finally, the water soluble phase PVA and NaCl were leached with circulating water at 40 °C for at least 72 h (water was changed every 6 h) until the sample weight was constant. The porous scaffolds were vacuum-dried before characterization.

2.4 Morphology Observation

The morphology of the PLA/PVA, PLA/PVA/NaCl, and the foamed PLA/PVA/NaCl scaffolds was observed under a scanning electron microscope (Quanta 200, FEI, USA) at 10 kV.

The scaffolds were immersed into liquid nitrogen to maintain the cell structure before fracturing in cross section. The fractured surfaces were coated with a thin layer of gold prior to microscopic observation. All samples used for SEM characterizations were leached unless specially illustrated.

2.5 Porosity Measurement

In accordance with the previous study, the porosity of the leached PLA scaffolds was calculated using the following Equation (1)[34]:

$$Porosity = \frac{V_{th}\rho - W_m}{V_{th}\rho} \times 100\% \quad (1)$$

where, ρ is the density of PLA; V_{th} is the volume of the foamed samples, which was measured with a density balance (DH-600 DahoMeter, Dongguan Hongtuo Instrument Co. Ltd); W_m is the weight of the sample determined by a electronic scale with high accuracy.

2.6 NaCl Contents and Its Residue after Leaching

The salt content of each blends was analyzed using a thermogravimetric analyzer (NETZCH TG 209, Germany). The samples were heated from 30 °C to 700 °C at a rate of 20 °C/min. To evaluate the influence of leaching time on the NaCl residue, porous scaffolds at different leaching time were burned in a muffle furnace (KSW-4-12, Shanghai Chongming Experimental Instrument Factory) at 700 °C to burn polymer matrix. Due to the decomposition temperature of salt is above 800 °C, after burning in the muffle furnace, the polymer matrix was burnt and NaCl particles was left. The remaining NaCl particles were measured by the aforementioned electronic scale and the content of NaCl was calculated according to the following Equation (2).

$$\Phi_{NaCl} = \frac{W_{NaCl}}{W_{sca}} \times 100\% \quad (2)$$

where, W_{NaCl} was the weight of residue after burning in the muffle furnace and W_{sca} was the weight of the scaffolds before leaching. Moreover, Laser diffraction particle size analyzer (LS 13320, USA) was employed to determine the size of NaCl particles before and after blending.

2.7 Biocompatibility Evaluations

2.7.1 Cell Culture

With human mesenchymal stem cell (hMSC) used as cell model, cell viability and proliferation were detected to investigate the basic biological performance of the prepared PLA scaffolds. The cells were placed into T75 tissue culture-treated polystyrene (TCPS) flasks (BD Falcon) at a density of 40,000/cm² after thawing and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 20% fetal bovine serum (FBS, WiCell), 1% penicillin–streptomycin (10, 000 U/mL, life technologies), 1% L-glutamine (200 mM, life technologies), and 1% MEM non-essential amino acids (life technologies). When cells reached 70% confluency, they were passaged using TrypLE Express (Life Technologies).

Scaffolds were first immersed into 70% ethanol for 15 min and then washed with PBS overnight, sterilized using ultra-violet (UV) light for 30 minutes on each side, washed with PBS three times, and then placed into 48-well TCPS plates that were pre-treated with polyHEMA to prevent cells from attaching to the TCPS plates. Cells were then seeded onto the scaffolds at a density of 20,000/well and cultured at incubator settings of 5% CO₂ and 37 °C. The media was refreshed every other day.

2.7.2 Live/dead Assay

To confirm the viability of cells cultured on the scaffolds, cells were stained with a Live/Dead Viability/Cytotoxicity Kit (Invitrogen) after culturing for 14 days. This kit allows simultaneous visualization of both live and dead cells. The stain utilizes 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 cellular membranes. A Nikon A1Rsi Ti-E confocal microscope was used to image the cells.

2.7.3 Cytoskeleton Study

The cytoskeleton organization of the cells was determined by phalloidin–tetramethyl-rhodamine B isothiocyanate (phalloidin–TMRho, Sigma) staining. For these assays, after 3 days of culture, cells were first washed with PBS twice and then fixed in 4% PFA diluted in PBS for 15 min at room temperature. Cells were then washed with PBS and permeabilized with 1% Triton-X in PBS for 6 min at room temperature. The cells were washed with PBS again and treated with 0.3 μ M phalloidin–TMRho with DAPI for 1 hour at room temperature. Samples were then washed with PBS and imaged using a fluorescence microscope.

2.7.4 Cell Proliferation

The proliferation of cells on the scaffolds was investigated after 3 days, 7 days and 14 days of seeding. The seeded samples were transferred to new well plates to ensure that the cells collected were not growing on the surface of the well plates. Then the transferred samples were washed twice with phosphate buffered saline (PBS), and 250 μ L Trypsin (Life Technologies) per well was used for 5 minutes at 37 °C to detach the cells. After incubation, the cells were collected and centrifuged at 1,000 rpm for 5 minutes. The supernatant was then aspirated and the

cells were resuspended in 600 μL of PBS and filtered prior to analysis. Data was acquired with an Accuri C6 (BD Biosciences).

3. RESULTS AND DISCUSSION

3.1 Morphology of PLA/PVA Blends

DSC analyses were conducted on neat PLA and PVA to determine the processing temperature of PLA/PVA blends. As shown in Figure 2(a), it can be observed that the melting temperature of PLA and PVA are 152 $^{\circ}\text{C}$ and 192 $^{\circ}\text{C}$, respectively. Based on our preliminary work, it was found that when the highest processing temperature was 200 $^{\circ}\text{C}$, the viscosity of PLA became very low and hard to solidify in air. Moreover, the color of PVA became yellow demonstrating the decomposition of PVA during processing at this 200 $^{\circ}\text{C}$. Therefore, the highest processing temperature profile was in between the two melting points of the PLA and PVA. Due to the good flow ability of PLA phase at the chosen temperature and the shear heat during processing, PVA phase was also easily processed.

The as extruded PLA/PVA blends via vane extruder were directly leached to investigate the blending efficiency of the vane extruder and determine the optimal leaching time. The SEM micrographes of PLA/PVA blends after leaching for different time periods were shown in Figure 2. Figure 2(b) shows the microstructure of the blend after extracting the PVA phase for 24 h. It was noticed that most PVA phase was leached out except a solid core. However, after leaching for 72 h, the solid pores disappeared with the presence of interconnected channels (as shown in Figure 2(c)). Most pores have size range from 1 to 5 μm , which was attributed to the formation of PLA/PVA co-continuous phases and the success removal of the water soluble PVA phase. Noticeably, pores with the diameter range of 10-30 μm were also observed in the blends. These

pores were resulted from the removal of unmelted PVA phase under the used processing temperature profile (which was slightly lower than the melting point of PVA as shown in the Figure 2(a)). The small pores are eligible to act as the micro-channels for the nutrition and waste transportation, while the large pores could facilitate cell habitation. However, it is obvious that the amount of large pores is not enough to resemble the extracellular matrix and to support cell attachment and migration. Hence, NaCl particles and subsequent foaming procedures were involved to further improve the porosity and regulate pore size.

3.2 The Effect of NaCl Content on the Scaffold Morphology

The scaffold morphology can be tuned by adjusting the salt concentration in the scaffolds. It can be seen from Fig. 3 that more large pores were introduced into the scaffolds after leaching as the increase of NaCl porogen content from 10 to 40%. The viscosity of the suspension is known to increase with the increase of solid particulate concentration. In this study, the maximum content of NaCl that can be attained was 40% with our machine, beyond which the extrude mixture lost their integrity and became hard to collect. With the addition of NaCl particles, all scaffolds showed a multimodal porous structure with distinct pore size and pore architecture after sufficient leaching. With the increase of NaCl content, the porous structure varies significantly. The macropores were created by the NaCl particles, while the micropore were generated by the co-continuous phase and the unmelted PVA. As the ratio of PLA to PVA was constant at 1 to 1 for four compounds, the difference between the scaffolds was ascribe to the concentration of NaCl particles. By varying the NaCl concentration, we are able to control the macropore size range from 25 μm to 90 μm . In addition, the inner wall of the pores possesses numerous micro channels which could assist nutrition transportation. Although the PVA polymer and NaCl particles as porogens introduced interconnected channels and macropores for the

scaffolds, there were still thick skin layers among the macropores to affect the interconnectivity of the scaffolds. They are undesirable for cell interactions and cell ingrowth. Moreover, the porosity of the scaffold was only 70% with the highest NaCl content. It is not optimal for tissue engineering applications. Therefore, further technique need to be used to enhance the scaffold porosity.

3.3 The Effect of Gas Foaming on Scaffold Morphology

To further enhance the porosity and interconnectivity of the scaffolds, supercritical CO₂ foaming was used to combine with the particulate leaching. In our experiments, we've tried compounds with various NaCl concentrations, and found that only compounds containing 10% and 20% NaCl particles could be foamed easily without causing sample collapse. When the NaCl content was further enhanced, the PLA/PVA/NaCl blends became difficult to foam and the porosity of the scaffolds was not increased. These was because the increment of non-melt polyvinyl alcohol and higher loading of NaCl particles make the CO₂ adsorption of the PLA matrix reduce, thus the foaming capacity of the blend was weakened. The morphology of the scaffolds containing 10% NaCl is shown as Figure 4 (a) and (b). Multimodal pore structure was observed in the foamed scaffolds. After extraction of NaCl particles, macropores with the size of hundreds of micrometers were left in the scaffolds. Besides, there were dense microscale pores after foaming distributed on the walls of the macropores, which were ascribed to the foaming of PLA matrix. However, those created macropores were not interconnected due to the limited content of NaCl in the foamed PLA/PVA/NaCl blend at the composition ratio of 50/50/10.

For the blends of PLA/PVA/NaCl with the composition ratio of 50/50/20, the macropores were connected (shown in Figure 4(c) and (d)). The number of the macropores was increased,

and it can be seen that there were still numerous micro pores on the wall of the macropores. Those micropores created the interconnections among macropores. The porosity and interconnectivity of the scaffold were achieved to be 93% and 96%, respectively. It was noticeable that the macropores created by NaCl particles after foaming were larger than their original size. This could be caused by the poor interphase bonding between NaCl particles and PLA matrix; The saturated gas expanded at the interphase and made the macropores larger than NaCl particles in the foaming process.

3.4 NaCl residues in the Scaffold

TGA was used to quantitatively determine the residual NaCl content in the blends. As shown in Figure 5(a), the NaCl content of the different blends were found to be 17.3%, 29.8%, 42.4%, 53.2% in weight of the blends, respectively. These results were closed to their theoretical values demonstrating that vane extruder has good mixing effect. To evaluate the effect of leaching time on the residual NaCl content, both unfoamed and foamed PLA scaffolds were selected and immersed in the 40 °C thermostatic water to extract the water soluble components, and then burned in muffle furnace at 700 °C for 6 h to obtain the residual NaCl components. Figure 5(b) shows the weight of the residual NaCl of unfoamed and foamed PLA scaffolds at different leaching time. As expected, the weight of residual NaCl decreased as the increase of leaching time. However, it is noticed that the foamed scaffolds needs less than 24 h to fully extract the water soluble NaCl particles in the scaffolds, while the the unfoamed scaffolds takes 72 h to achieve 0% NaCl residual. The fast leaching speed of famed scaffolds was attributed to their high porosity and interconnectivity, as suggested in the SEM results.

3.5 Morphology of NaCl Particles after Processing

High shear stress generated by conventional screw extruder could cause sharp attrition of the salt particles during blending especially in the melting zone of the extruder. In this study, instead of applying shear stress, we applied strong elongational stress to the melt compounds via the vane extruder we developed. In this section, we analyzed in detail of the effect vane extrusion on the size change of NaCl particles, the morphology of the PLA/NaCl blend system, and the underlining mechanism.

First, we compared the effect of three different compounding methods on the morphology and particle size change of NaCl particles. Figure 6 (a) depicts the NaCl particles before blending. Figure 6 (b), (c) and (d) presented the NaCl particles morphologies and size distributions after compounding via vane extruder, twin screw extruder, and triple screw extruder, respectively. It can be seen that before mixing, the NaCl powder is composed of uniformly distributed large particles with average particle size of 120 μm . However, after going through the extruder, the particle size showed a bimodal distribution, which indicate that some NaCl particles have been broken apart while some remained their size. By comparing the fraction of the small NaCl particles and the large NaCl particles, one can easily tell that the vane extruder performed much better than twin screw and triple screw extruders. The average NaCl particle size was over 90 μm after blending via vane extruder. Moreover, we also found that the particles changed from cubic shape to round shape after compounding. The pliable shape of NaCl particles could result in round pores which can better resemble the porous structure in native extracellular matrix and should be favorable for cell migration.

We compared the morphologies of as extruded PLA/NaCl blends with 40% NaCl concentration using twin screw extruder (TSE) and vane extruder (VE), to reveal the effect of elongational flow on the phase morphology of PLA/NaCl blends. As shown in Figure 7, we

found that it is already hard to distinguish individual NaCl particles from the TSE-extruded sample, and the interphase between NaCl and PLA was not clear as well, which means that NaCl particles may have been warped by PLA matrix, and it would cause difficulties in the leaching step. However, the VE-extruded sample clearly showed lots of NaCl particles loosely embedded within the polymer matrix as indicated by the red circles. Therefore, the composites prepared via vane extrusion would give better porosity, pore size uninformative and pore interconnectivity to the final scaffolds produced. These assumptions from the phase morphology have been confirmed by the resulted presented in the above sections.

Figure 8 depicts the schematic illustration of the flow fields and the NaCl particle flow pattern differences in steady shear flow (twin screw extrusion) and volume elongational flow (vane extrusion) in order to reveal the underlining mechanism. As shown in the figure, in the case of steady shear flow, the velocity gradient is perpendicular to the flow direction, hence the NaCl particles were inclined to move in a strong shear layer and rotate by themselves; they subject to strong shear force which would tend to broke them apart. Furthermore, there are many channels on the screws of twin screw extruder, so that NaCl particles would intermittently been pushed by the ridges and squeezed by the contact of ridges and barrel, which further caused the distruction of NaCl particles. However, for the volume elongation flow field, as shown in the figure, the velocity gradient formed a small angle with the flow direction, which means that the velocity variation is mostly along the flow direction, thus the polymer molecular chains were much easier to move along the flow direction in elongational flow compared with in shear flow[35]. Therefore, the NaCl particles subjected to a stretched force along the flow direction which facilitated the moment of NaCl particles. This is also the reason that much higher NaCl concentration could be achieved with vane extruder. Frankly, NaCl destruction did happen as

well in vane extrusion as shown in Figure 6 (b). The broken of small amount of NaCl particles was mostly caused by the friction between siding plate and the barrel. However, majority of NaCl particles could preserve their original size when been compounded by vane extruder. These results emphasized the significant advantages of the vane extrusion over the conventional screw extrusion.

3.6 Cell Viability and Proliferation

Human mesenchymal stem cells were cultured on the scaffolds prepared with the addition of 10% and 20% NaCl particles for up to 14 days to verify the biocompatibility of the scaffold prepared in this study. The PLA/PVA/NaCl (50/50/10) scaffolds have an average porosity of 85%, and the average porosity of PLA/PVA/NaCl (50/50/20) scaffolds was 93%. Figure 9 shows the cell viability results and the corresponding cytoskeleton results were shown as the inset images. It can be seen that most cells all the scaffolds were alive indicated by green fluorescence and only a few dead cells were observed which were presented in red fluorescence dots, demonstrating that the scaffolds with different porosity all have good biocompatibility. However, cells on the foamed PLA/PVA/NaCl (50/50/20) scaffolds were more stretched than those on the foamed PLA/PVA/NaCl (50/50/10) scaffolds, demonstrating a more flourishing living state of cells on the scaffolds. These results suggested that the scaffolds have higher porosity and better pore interconnectivity are more favorable for cell to survive.

Generally, mammalian cells on the scaffolds undergo a cell adhesion process including substrate attachment, spreading and cytoskeleton development [36]. The cytoskeleton organization of hMSCs on the scaffolds was assessed by actin staining in this study. Cell morphology and the organization of actin structure were compared. Cells grown on the

PLA/PVA/NaCl (50/50/10) scaffold displayed a narrow shape, which is a limited adherent and spreading of cells on the scaffolds. By contrast, cells on PLA/PVA/NaCl (50/50/20) scaffolds showed a more spreading morphology. As a result, cells showed a much stronger and organized stretched actin bundles on the PLA/PVA/NaCl (50/50/20) scaffolds. However, cells exhibiting a narrow shape on PLA/PVA/NaCl (50/50/10) scaffolds displayed much fewer organized actin stress fibers. These results indicated that the scaffolds with higher porosity and interconnectivity performed better in terms of cell attachment and cell morphology.

To further investigate the cell proliferation on the scaffolds, flow cytometry was carried out to test the cell population on the scaffolds. Figure 10 displays the results of cell proliferation on the scaffolds. At day 3 and day 7, there were more cells on PLA/PVA/NaCl (50/50/20) than their counterparts. At day 14, the cell population on PLA/PVA/NaCl (50/50/20) was significantly higher than that of PLA/PVA/NaCl (50/50/10). These results further indicated that the high porosity, interconnected structure of scaffolds was beneficial for cell proliferation and cell growth, which was consistent with the cell viability and cytoskeleton results. It was because the interconnected porous structure in the scaffolds was necessary for the transportation of nutrition and the interactions between cells, and the high porosity facilitated the cell growth and migration. Therefore, the scaffolds prepared in this study have potential to be used in tissue engineering applications.

4. CONCLUSIONS

In this study, we proposed a novel method to prepare porous PLA scaffolds by a hybrid method of melt blending, supercritical CO₂ foaming, and particulate leaching based on self-developed vane extruder. The compounding effects of vane extruder were investigated in detail

by comparing with conventional screw extrusion technique. Different from screw extrusion which is based on shear flow mixing, the vane extrusion was dominated by elongational flow mixing. The special mixing mechanism ensured the NaCl particles to retain their original size and facilitated mixing and flow of the compounds, proved that the vane extruder was more suitable for precisely controlling the macropores size of the scaffolds. Using the proportion optimized PLA/PVA/NaCl (50/50/20) composites, scaffolds with average porosity of 93% and average pore interconnectivity of 96% were successfully fabricated after CO₂ foaming and particulate leaching. Moreover, the porous structure created was consisted of multimodal pores, the interconnection channels were generated by the water soluble PVA phase. The preliminary cell culture results demonstrated that the scaffolds with high porosity and pore interconnectivity had good biocompatibility and were highly potential to be used in tissue engineering applications.

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6. REFERENCES

- [1] Langer R, Vacanti JP. Tissue Engineering. Science. 1993;260:920-6.
- [2] Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. Biomaterials. 2000;21:2529-43.

- [3] Mikos AG, Bao Y, Cima LG, Ingber DE, Vacanti JP, Langer R. Preparation of poly(glycolic acid) bonded fiber structures for cell attachment and transplantation. *J Biomed Mater Res.* 1993;27:183-9.
- [4] Pham QP, Sharma U, Mikos AG. Electrospinning of polymeric nanofibers for tissue engineering applications: A review. *Tissue Eng.* 2006;12:1197-211.
- [5] Rajzer I. Fabrication of bioactive polycaprolactone/hydroxyapatite scaffolds with final bilayer nano-/micro-fibrous structures for tissue engineering application. *J Mater Sci.* 2014;49:5799-807.
- [6] Mikos AG, Sarakinos G, Leite SM, Vacanti JP, Langer R. Laminated 3-Dimensional Biodegradable Foams for Use in Tissue Engineering. *Biomaterials.* 1993;14:323-30.
- [7] Mikos AG, Thorsen AJ, Czerwonka LA, Bao Y, Langer R, Winslow DN, et al. Preparation and Characterization of Poly(L-Lactic Acid) Foams. *Polymer.* 1994;35:1068-77.
- [8] Hou QP, Grijpma DW, Feijen J. Porous polymeric structures for tissue engineering prepared by a coagulation, compression moulding and salt leaching technique. *Biomaterials.* 2003;24:1937-47.
- [9] Nam YS, Park TG. Biodegradable polymeric microcellular foams by modified thermally induced phase separation method. *Biomaterials.* 1999;20:1783-90.
- [10] Lee KWD, Chan PK, Feng XS. Morphology development and characterization of the phase-separated structure resulting from the thermal-induced phase separation phenomenon in polymer solutions under a temperature gradient. *Chem Eng Sci.* 2004;59:1491-504.
- [11] Mi HY, Jing X, Salick MR, Cordie TM, Peng XF, Turng LS. Morphology, mechanical properties, and mineralization of rigid thermoplastic polyurethane/hydroxyapatite scaffolds

- for bone tissue applications: effects of fabrication approaches and hydroxyapatite size. *J Mater Sci.* 2014;49:2324-37.
- [12] Mooney DJ, Baldwin DF, Suh NP, Vacanti JP, Langer R. Novel approach to fabricate porous sponges of poly(d,l-lactic-co-glycolic acid) without the use of organic solvents. *Biomaterials.* 1996;17:1417-22.
- [13] Barry JJA, Silva MMCG, Cartmell SH, Guldberg RE, Scotchford CA, Howdle SM. Porous methacrylate tissue engineering scaffolds: using carbon dioxide to control porosity and interconnectivity. *J Mater Sci.* 2006;41:4197-204.
- [14] Dehghani F, Annabi N. Engineering porous scaffolds using gas-based techniques. *Curr Opin Biotech.* 2011;22:661-6.
- [15] Rouholamin D, Smith PJ, Ghassemieh E. Control of morphological properties of porous biodegradable scaffolds processed by supercritical CO₂ foaming. *J Mater Sci.* 2013;48:3254-63.
- [16] Leong KF, Cheah CM, Chua CK. Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs. *Biomaterials.* 2003;24:2363-78.
- [17] Fierz FC, Beckmann F, Huser M, Irsen SH, Leukers B, Witte F, et al. The morphology of anisotropic 3D-printed hydroxyapatite scaffolds. *Biomaterials.* 2008;29:3799-806.
- [18] Melchels FPW, Feijen J, Grijpma DW. A poly(D,L-lactide) resin for the preparation of tissue engineering scaffolds by stereolithography. *Biomaterials.* 2009;30:3801-9.
- [19] Iannace S, Di Maio E, Nicolais L. Preparation and characterization of polyurethane porous membranes by particulate-leaching method. *Cell Polym.* 2001;20:321-38.

- [20] Oh SH, Kang SG, Kim ES, Cho SH, Lee JH. Fabrication and characterization of hydrophilic poly(lactic-co-glycolic acid)/poly(vinyl alcohol) blend cell scaffolds by melt-molding particulate-leaching method. *Biomaterials*. 2003;24:4011-21.
- [21] Washburn NR, Simon CG, Tona A, Elgendy HM, Karim A, Amis EJ. Co-extrusion of biocompatible polymers for scaffolds with co-continuous morphology. *J Biomed Mater Res*. 2002;60:20-9.
- [22] Sarazin P, Roy X, Favis BD. Controlled preparation and properties of porous poly(L-lactide) obtained from a co-continuous blend of two biodegradable polymers. *Biomaterials*. 2004;25:5965-78.
- [23] Reignier J, Huneault MA. Preparation of interconnected poly(epsilon-caprolactone) porous scaffolds by a combination of polymer and salt particulate leaching. *Polymer*. 2006;47:4703-17.
- [24] Aronin CEP, Cooper JA, Sefcik LS, Tholpady SS, Ogle RC, Botchwey EA. Osteogenic differentiation of dura mater stem cells cultured in vitro on three-dimensional porous scaffolds of poly(epsilon-caprolactone) fabricated via co-extrusion and gas foaming. *Acta Biomater*. 2008;4:1187-97.
- [25] Jing X, Mi HY, Cordie T, Salick M, Peng XF, Turng LS. Fabrication of Porous Poly(e-caprolactone) Scaffolds Containing Chitosan Nanofibers by Combining Extrusion Foaming, Leaching, and Freeze-Drying Methods. *Ind Eng Chem Res*. 2014;53:17909-18.
- [26] Mi HY, Jing X, Salick MR, Turng LS, Peng XF. Fabrication of thermoplastic polyurethane tissue engineering scaffold by combining microcellular injection molding and particle leaching. *J Mater Res*. 2014;29:911-22.

- [27] Cui ZX, Zhao HB, Peng YY, Kaland M, Turng LS, Shen CY. Morphology and Properties of Porous and Interconnected Poly(epsilon-caprolactone) Matrices Using Solid and Microcellular Injection Molding. *J Biobased Mater Bio*. 2012;6:259-68.
- [28] Kramschuster A, Turng LS. An Injection Molding Process for Manufacturing Highly Porous and Interconnected Biodegradable Polymer Matrices for Use as Tissue Engineering Scaffolds. *J Biomed Mater Res B*. 2010;92B:366-76.
- [29] Stary Z, Machui F, Munstedt H. Elongational creep experiments - A new method for investigations of morphology development in polymer blends. *Polymer*. 2010;51:3744-52.
- [30] Qu JP, Liu LM, Tan B, Liu SR, Chen HX, Feng YH. Effect of dynamical converging channels on fiber organization and damage during vane extrusion of sisal fiber-reinforced polypropylene composites. *Polym Composite*. 2012;33:185-91.
- [31] Qu JP, Chen HZ, Liu SR, Tan B, Liu LM, Yin XC, et al. Morphology study of immiscible polymer blends in a vane extruder. *J Appl Polym Sci*. 2013;128:3576-85.
- [32] Zhang HC, Huang JT, Yang L, Chen RY, Zou W, Lin XK, et al. Preparation, characterization and properties of PLA/TiO₂ nanocomposites based on a novel vane extruder. *Rsc Adv*. 2015;5:4639-47.
- [33] Qu JP, Zhao XQ, Li JB, Cai SQ. Power consumption in the compacting process of polymer particulate solids in a vane extruder. *J Appl Polym Sci*. 2013;127:3923-32.
- [34] Mi HY, Salick, R. M., Jing, X., Jacques, R. B., Wendy, C. C., Peng, X. F., Turng, L., S. Characterization of thermoplastic polyurethane/polylactic acid (TPU/PLA) tissue engineering scaffolds fabricated by microcellular injection molding. *Materials Science and Engineering: C*. 2013;33:4767-76.

- [35] Qu JP, Yang ZT, Yin XC, He HZ, Feng YH. Characteristics Study of Polymer Melt Conveying Capacity in Vane Plasticization Extruder. *Polym-Plast Technol.* 2009;48:1269-74.
- [36] Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. *Biomaterials.* 2003;24:4385-415.

Figure Captions

Fig. 1 Schematic illustration of the vane extruder

Fig. 2 (a) DSC curves of neat PLA and PVA, respectively; SEM results of (b) PLA/PVA blend after leaching for 24 h, (c) PLA/PVA blend after leaching for 72 h, (d) higher magnifications of the dashed red square part in image (c).

Fig. 3 Morphology of PLA porous scaffolds as a function of NaCl content: (a) 10 vol%; (b) 20 vol%; (c) 30 vol%; (d) 40 vol%. The PLA/PVA composition ratio is kept at 50/50 (v/v) for all the blends (the scale bars in the images and insert images are 100 μm and 20 μm , respectively)

Fig. 4 Morphology of PLA scaffolds prepared by combination of particulate leaching and supercritical CO_2 foaming, (a) and (b) the PLA/PVA/NaCl volume composition ratio is 50/50/10; (c) and (d) the PLA/PVA/NaCl volume composition ratio is 50/50/20.

Fig. 5 (a) TGA curves of PLA/PVA/NaCl blends with different NaCl contents; (b) The NaCl residues of unfoamed and foamed PLA/PVA/NaCl (50/50/20) components at different leaching time.

Fig. 6 The morphology of NaCl particle before and after processing by different flow field. (a) original NaCl particles before processing, (b) NaCl particles processed by vane extruder, (c) NaCl particles processed by twin extruder and (d) NaCl particles processed by triple screw extruder.

Fig. 7 Morphologies of PLA/NaCl blends with 40% of NaCl prepared via (a) twin screw extruder; and (b) vane extruder.

Fig. 8 The schematic diagram of the movement of salt particles under different flow field: (a) steady shear flow and (b) volume elongational flow.

Fig. 9 Day 14 live/dead cell assays showing hMSCs cultured on (a) PLA/PVA/NaCl (50/50/10) and (b) PLA/PVA/NaCl (50/50/20) porous scaffolds (live cells showed green fluorescence and dead cells produced red fluorescence). Cytoskeleton development of cells are shown in the insert images (the red color indicated actin stress fibers and purple color represented the cell nuclei).

Fig. 10 Cell proliferation results of hMSCs on the scaffolds ($p < 0.05$).

1: Hopper; 2: Stator; 3: Vane; 4: Baffle; 5: Heater; 6: Rotor; 7: Die; 8: Extrudate

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