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Porous nanostructured poly-L-lactide scaffolds prepared by phase inversion using supercritical CO_2 as a nonsolvent in the presence of ammonium bicarbonate particles

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ABSTRACT

Supercritical fluid technology has been utilized in the development of tissue engineering scaffolds. However, it results in some problems, such as the poorly interconnected pores and the inability to load growth factor due to the salt leaching process for removal of the solid porogen. In this study, ammonium bicarbonate (AB) particles were used as a porogen and mixed uniformly with poly-L-lactide (PLLA) solution. Supercritical CO_2 was used to immerse and flush through the resulting compound to allow the occurrence of phase inversion, subsequently generating a nanofibrous network. As the decomposition temperature of AB crystals is 36 °C, the temperature of the CO_2 was increased to 40 °C to decompose the porogen, and the decomposition products were removed by washing with CO_2. The resulting PLLA scaffolds possessed both large pores and micro pores with a controllable pore size, a high porosity (>95%), an interconnected structure, a nanofibrous network, good mechanical properties (compressive strength up to 100 kPa), and a very low organic solvent residue (12 ppm). The results of Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) measurements indicated that the molecular structure and physical state of PLLA were not changed after supercritical processing. The results reveal that the application of AB particles as a porogen in the supercritical phase inversion process is feasible to produce tissue engineering scaffolds with a high-performance.

1. Introduction

PLLA is a biodegradable aliphatic polyester derived from renewable resources; it has been extensively studied and used in the area of industrial packaging and biomedical applications such as surgical implants, controlled drug delivery devices, and scaffolds for tissue engineering [1–3]. PLLA has been processed in different forms, such as particles, fibers and membranes, to be used in tissue engineering applications [4–6]. In these applications, PLLA scaffolds have been described as delivery systems capable of carrying active agents or bio-molecules and growth factors [7,8].

As an important part of tissue engineering, three-dimensional (3D) scaffolds play a pivotal role in cell seeding, cell proliferation, and new tissue formation [9]. One of the most important stages of building scaffolds is the design and processing of a porous 3D structure with high porosity, high interconnectivity, and uniform distribution of the pores. A variety of processing techniques have been developed and include particle leaching, gas foaming processes, thermally induced phase separation, and nonsolvent-induced phase inversion [10–13]. The main disadvantages of these methods are the use of organic solvents and the high temperatures required. Since the presence of residual organic solvents is being rigorously controlled by the international safety regulations, it is necessary to warrant the complete removal of these substances. Therefore, supercritical fluid technology appears to be an interesting alternative to the traditional processing method [14,15].

Nonsolvent-induced phase inversion technology has been widely used for fabricating porous polymer scaffolds [16,17]. During the process of fabricating porous polymer scaffolds, a nonsolvent was added into a homogeneous polymer solution, which leads to the separation of the polymer solution into a polymer-rich phase and a polymer-lean phase. The polymer-rich phase develops a continuous matrix while the polymer-lean phase mainly

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consisting of the solvent leads to generate porous tunnels within the matrix, thus forming a continuous, interconnected porous structure [18]. The supercritical CO_2 can be used as a nonsolvent instead of organic solvents, and this adds several advantages to the process. One of the most important advantages of using supercritical CO_2 is the fact that the final structure of the product can be tailored by simply tuning the operating parameters of pressure and temperature [19]. Moreover, when CO_2 is used as a nonsolvent, the collapse of the structure will not occur during the drying process due to the absence of a liquid–vapor interface, and the porous structure is free of any residual solvents [20].

Recently, a process in which supercritical CO_2 replaces the liquid nonsolvent for phase separation has been proposed [21], but the porous scaffolds prepared by the gas foaming techniques using supercritical CO_2 have a relatively closed pore network [22,23]. To overcome this issue, an additional porogen (sodium chloride particles) was incorporated into the compression molding of polymer before gas foaming. After gas foaming, the porogen was leached out by incubation in water for 48 h, thus creating a scaffold with an interconnected pore network [24]. However, the leaching of the porogen is a major disadvantage of this process, since it results in loss of the majority of any incorporated growth factors [25]. If the porogen in the scaffold was replaced by AB particles, the problem of the growth factor loss would be resolved, since the AB particles would break down at temperatures above 36 °C.

In this work, we attempted to produce a porous nanostructured PLLA scaffold with interconnected pores by phase inversion, using supercritical CO_2 as a nonsolvent in the presence of AB particles. Briefly, the AB particles were added to the PLLA solution and mixed vigorously, and then the polymer/salt/solvent paste was cast into the steel mold. The resulting compound was immersed in supercritical CO_2 to allow the occurrence of phase separation. Afterwards, the system was flushed with supercritical CO_2 for another 2 h to generate a nanofibrous network. As the decomposition temperature of AB crystals is 36 °C, the temperature of the CO_2 was increased to about 40 °C to decompose the porogen, and the decomposition products were removed by washing with CO_2.

2. Materials and methods

2.1. Materials

PLLA with an inherent viscosity of 3.5 dl/g (0.1% (wt/v) in chloroform, 25 °C) was purchased from the Jinan Daigang Biological Co., Ltd. (Jinan, China). AB and dichloromethane (99.8% purity) were bought from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and CO_2 of 99.9% purity was purchased from the Rihong Air Products Co., Ltd. (Xiamen, China). All materials were used as received.

2.2. Methods

2.2.1. Scaffold preparation

PLLA was firstly dissolved in different solvents, namely dichloromethane, 1,4-dioxane, and dichloromethane/1,4-dioxane (1:1, v: v); the solution was stirred until it became homogeneous. Then, AB particles (size range 300–600 μm) were added into the solution and mixed vigorously. The AB/PLLA (w:w) ratios ranged from 10 to 40 and the concentration of PLLA was set as 10% (wt/v). The polymer/salt/solvent paste was cast into the steel mold, and then the resulting compound with a diameter of 2.0 cm and height of 5.0 mm was placed in the high-pressure vessel. Supercritical CO_2 was pumped into the high-pressure vessel to the desired pressure (15 MPa) and temperature (35 °C). After treatment for 2 h, the vessel was flushed with fresh CO_2 with a constant flow rate (approximately 5 g/min) for 2 h to remove the organic solvent. During this process, the pressure and temperature were kept constant. Then, the system was rapidly depressurized to atmospheric pressure within 2 min. The temperature in the high-pressure vessel was increased to 40 °C, and the scaffold was flushed at a constant flow rate (approximately 5 g/min) until the AB particles were completely decomposed.

2.2.2. Scanning electron microscopy (SEM)

PLLA scaffolds were fractured in liquid nitrogen. Samples were sputter coated with gold for 180 S (Ion Sputter E-1010, Hitch, Japan). The overall scaffold structure was analyzed using a scanning electron microscope (SEM S-4800, Hitch, Japan).

2.2.3. Scaffold porosity

The "void space" of the scaffold was represented by the porosity (ε); the scaffold porosity was calculated by the equation below.

$$\varepsilon = 1 - \frac{\rho_s}{\rho_p}$$

where ρ_s is the density of the scaffold (scaffold weight/scaffold volume, g/cm^3) and ρ_p is the density of as received PLLA (1.24 g/cm^3).

2.2.4. Mechanical tests

The compressive mechanical properties of the scaffolds were measured on a UTM 6102 (Suns Co., Ltd., Shenzhen, China) equipped with a 0.1 kN load cell at room temperature. Cylindrical samples with a diameter of 2.0 cm and height of 5.0 mm were used. The cross-head speed was set at 1.0 mm/min. The compressive strength at 25% strain was determined by the ISO 604:2002.

2.2.5. Solvent residue analysis

Dichloromethane and 1, 4-dioxane residue was measured using a headspace sampler (G1888, Agilent Technologies, USA) coupled to a gas chromatograph (GC) interfaced with a flame ionization detector (6890N, Agilent Technologies, USA).

2.2.6. FTIR analysis

Samples of approximately 1 mg were pressed into a pellet with 200 mg of potassium bromide, and FTIR spectra were collected in continuous scan mode (wavelength range: 4000–400 cm^{-1}) and at a resolution of 10 cm^{-1} on a Nicolet iS10 system (Thermo, USA).

2.2.7. XRD analysis

XRD analysis was carried out using a PANalytical X'Pert PRO. The measurement was performed in the range of 10–35° with a step size of 0.013° in 2θ using Cu Kα radiation as the source.

2.2.8. Differential scanning calorimetry (DSC) analysis

The melting behavior of PLLA scaffolds was investigated by employing a Netzsch DSC 200F3 differential scanning calorimeter. The calibration was performed with indium and all tests were carried out in ultra-pure nitrogen as the purge gas. Samples were heated from 30 °C to 200 °C at a rate of 10 °C/min.

2.2.9. Statistical analysis

All date presented are expressed as mean ± standard deviations (SD). ANOVA single factor analysis were conducted, with the level of statistical significance set at $P < 0.05$. Every experiment was carried out in triplicate ($n = 3$).

3. Results and discussion

3.1. Surface morphology

In this work, the possibility of preparing PLLA scaffolds for tissue engineering using a supercritical assisted phase inversion technique in the presence of AB particles were evaluated.

Using conventional methods to produce scaffolds, in order to improve the porosity and interconnectivity of the scaffold, researchers have modified the process and proposed a hybridization of the supercritical fluids process with particulate leaching [26,27]. They introduced an insoluble porogen into the polymeric solution, such as D-fructose or sodium chloride particles. In order to eliminate the porogen in this process, the whole scaffold must be immersed in water or organic solvent for a long time to dissolve the particles [28]; this may lead to the loss of growth factor in the scaffold [25]. Sometimes, the samples were immersed into an excess amount of hot water (90 °C) to decompose the porogen, which may lead to the denaturation of the growth factor in the scaffold [29].

In this study, we selected AB particles of controlled size as the porogen, since it is insoluble in supercritical CO_2 and will break down at temperatures above 36 °C [29]. During the supercritical CO_2 processing, the temperature was kept at 35 °C. After the formation of a nanofibrous network, the AB particles were removed by increasing the temperature to 40 °C, to obtain an interconnected nanofibrous structure. This avoids the leaching process, which will denature the bioactive substances loaded in the scaffolds. According to the SEM images of PLLA scaffolds prepared under different pressures (as shown in Fig. S1 of Supplementary Material), it is found that the influence of pressures on the surface morphology of scaffolds was not significant; hence the operating pressure was set as 15 MPa in this study.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.supflu.2013.02.020.

Fig. 1 represents the optical image of the PLLA scaffold, which indicates that the shape and 3D structure of the tissue to be substituted could be reproduced accurately by the supercritical-CO_2-assisted phase inversion process. In the phase inversion process, the properties of the final porous structure are mainly controlled by the precipitation temperature, the pressure of the vessel, the flow rate of CO_2, and the concentration of the casting solution [23]. The role of the organic solvent in the morphology of the matrixes obtained was discussed only a few times in the supercritical-CO_2-assisted phase inversion technique. By the phase inversion with supercritical CO_2 as a nonsolvent, chitosan may form a dense membrane from formic acid, a macro-void structure with a dense layer on top from HFIP solution and a homogeneous scaffold from acetic acid solution [19]. Matsuyama [20] investigated the influence of four kinds of organic solvents on the porous cellulose acetate membranes; they found that as the mutual affinity decreased between solvent and supercritical CO_2, the membranes porosity and average pore size near the center position increased. These results reveal that the influence of different solvents on the morphology of scaffolds in the phase inversion process is significant.

Fig. 2 shows the SEM images of the cross section of the scaffolds prepared from different organic solvents and the images of local magnification. From these images, it is noticeable that the structure obtained was strongly dependent on the solvent used. When PLLA was precipitated from the dichloromethane solution, a micro pore structure was obtained; pore size ranged from 10 μm to 50 μm, and the pore wall was thick and dense. However, the scaffolds obtained from the 1,4-dioxane solution presented a nanofibrous network structure, which was not observed in the PLLA scaffolds precipitated from dichloromethane. Furthermore, the scaffolds processed from the 1,4-dioxane solution presented

Fig. 1. Optical image of PLLA scaffold prepared by supercritical phase inversion process.

a homogeneous nanofibrous structure and a cell size below 10 μm, with both micro and nanometer pores. When the mixture solution of dichloromethane/1,4-dioxane was used, the scaffolds obtained presented a homogeneous micro cell structure; the inner pore wall was rough and consisted of nanoscale cells. It was also observed that the size of the large pores formed by the porogen was identical to that of the AB particles.

The formation mechanism of the porous scaffold in this study can be proposed as follows. After the polymer/salt/solvent compound was placed in the supercritical CO_2, some amounts of the organic solvents were extracted from the compound by the supercritical CO_2 and the concentration gradient of polymer was formed. Then, the supercritical CO_2 was diffused into the sample, thus inducing a phase separation and generating a polymer-rich phase and a polymer-lean phase. When the supercritical CO_2 came out of the scaffold, the organic solvents in the scaffold were extracted by the supercritical CO_2. After the flushing of scaffold by CO_2, the dried nanofibrous structure was obtained. The process involves a ternary mixture (solvent, nonsolvent and polymer) interaction [30], the porous structure was influenced by the interaction between liquid–liquid phase and liquid–solid phase [31]. Such as, when the phase inversion takes place at a dichloromethane solution, the lower viscosity of the solution results in a slower phase inversion rate, which providing a longer phase inversion time to form larger pores. At the same time, the cellular structures may be influenced by the addition of porogen. On the one hand, the addition of porogen could in favor of the heterogeneous nucleation of the polymer-lean phase, which would form the structure with smaller pores [32]; On the other hand, the increase in viscosity by the addition of porogen may decrease the crystallization rate and provide a longer phase inversion time to form larger pores [33].

The formation of micro pores on the scaffolds prepared from the dichloromethane solution is related to the low boiling point (40 °C)

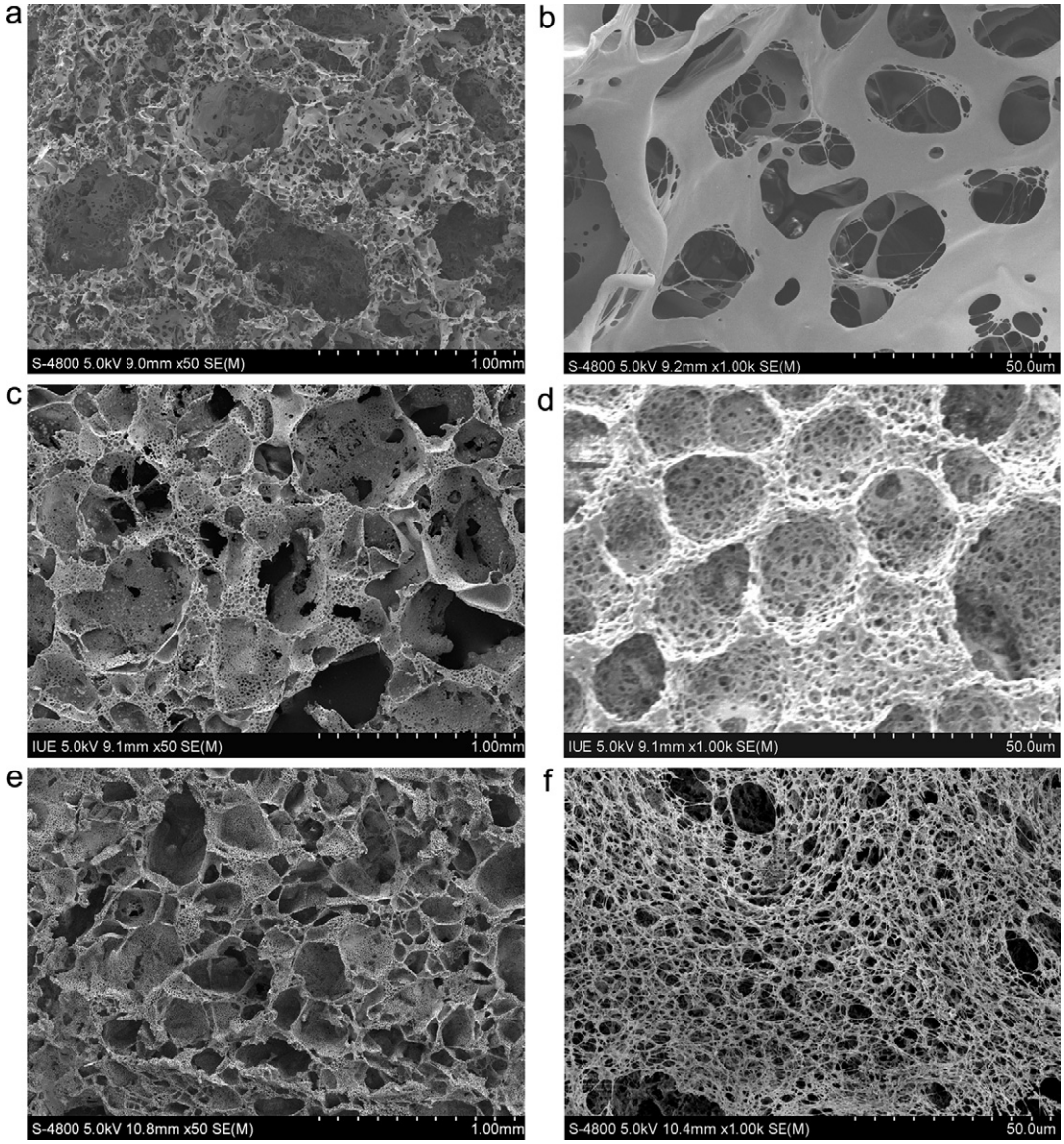

Fig. 2. SEM images of the scaffolds prepared from different organic solutions at 35 °C and 15.0 MPa: dichloromethane (a) 50×, (b) 1000×, dichloromethane/1,4-dioxane (1:1) (c) 50×, (d) 1000×; 1,4-dioxane (e) 50×, (f) 1000×.

of the organic solvent, as this is close to the operating temperature (35 °C). The solubility of dichloromethane in the supercritical CO_2 is higher than that of 1,4-dioxane, which will favor the phase inversion process because a higher affinity of the solvent to the supercritical CO_2 will cause phase inversion and precipitation of the polymer with a porous structure. Conversely, 1,4-dioxane solution is an organic solvent with a relatively high boiling point of 101 °C, which is above the operating temperature. A lower solvent affinity, that is, a higher square solubility parameter difference, will result in a decrease in the pore size of the scaffold, and this is favorable for the formation of nanofibers. At the same time, the viscosity of the solution will influence the average pore size. Since the viscosity of dichloromethane is lower than that of 1,4-dioxane, the coarsening of the micro droplets of the polymer-lean phase, which may lead to larger pores, is favored [34,35]. These results demonstrate that completely different morphologies can be obtained from precipitation with different solvents.

It has been proved that cell behavior can be mainly determined by the substrate that they are cultured on, and the understanding of interaction mechanism between cell and nano-topography structures could provide valuable information for the design of tissue engineering [36]. The nanofibrous structure may influence the cell differentiation, migration and protein absorption [37,38]. Three kinds of nano-topography structures have been prepared by the supercritical phase inversion technology in this experiment, it may very meaningful to induce cell growth on the scaffolds produced, to verify the effect of the mechano-topographic modulation structure of the scaffold on the preferential differentiation of the cells on next step.

3.2. Porosity and mechanical strength of scaffold

In general, an increase in porosity will decrease the compressive strength of the scaffold, and vice versa. Hence, it is important to deal with such relations well as porosity-porogen/PLLA ratio and compressive strength – porogen/PLLA ratio. In this study, different ratios of AB to PLLA were tested to find a suitable ratio to achieve a high porosity and acceptable mechanical strength. Fig. 3 shows the influence of the different ratios of AB to PLLA on the porosity and mechanical strength of the scaffold. When the mass ratio of

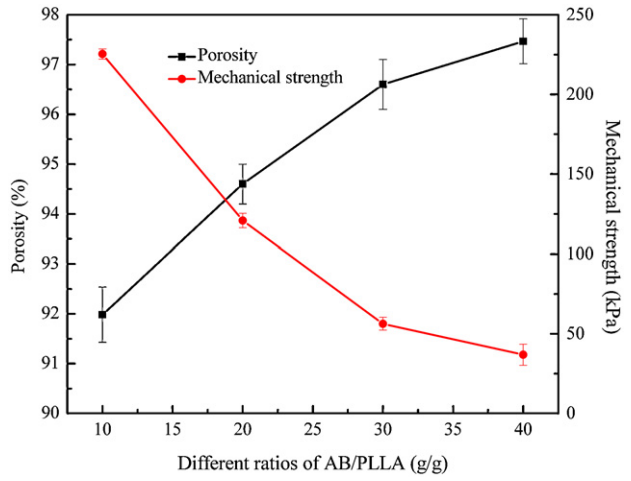

Fig. 3. The influence of the different ratios of AB to PLLA on porosity and mechanical strength (at 25% strain).

AB to PLLA increased from 10 to 40, the porosity of the scaffold was significantly increased ($P < 0.05$), from 92.0% ± 0.55 to 97.5% ± 0.45; however, the mechanical strength was significantly decreased from 225.4 kPa ± 3.16 to 36.8 ± 6.6 kPa ($P < 0.05$). The requirement of mechanical resistance and porosity depends on the organs to be regenerated; for instance, 100 kPa is necessary for bone scaffolds [39]. At the same time, the SEM image (Fig. 4) shows that the average pore size increased with the increase in AB/PLLA ratio. These results reveal that scaffolds with desirable properties for different organs could be obtained by adjusting the ratios of AB to PLLA.

3.3. Solvent residue and analysis

Without further treatment, the dichloromethane residue in the scaffold was only 12 ppm; this is much lower than the limit of the USP 467 Pharmacopeia (600 ppm). Although organic solvents were employed, the use of supercritical CO_2 allowed their complete removal; as the supercritical CO_2 has good diffusivity and mass transfer properties, its ability to diffuse and penetrate into the bulk of the 3D matrix allowed the complete extraction of the organic solvents [21]. Reverchon and Ji [28,40] also reported that supercritical CO_2 technology could produce a scaffold with an organic residue of less than 5 ppm and 10 ppm, respectively. However, the residual dichloromethane content of the scaffolds fabricated using the electrospinning method was 365 ppm after freeze drying for seven days [41], which is much higher than that of scaffolds prepared by supercritical CO_2 technology without further downstream processing. These results indicate that supercritical CO_2 technology is an effective method of producing a scaffold with little or no organic residue.

3.4. FTIR analysis

The FTIR spectra of the as-received PLLA and PLLA scaffolds are shown in Fig. 5. In these spectra, the PLLA characteristic bands are present; for example, the band at 1762 cm^{-1} was attributed to C=O stretching, the bands at 2949 and 2997 cm^{-1} were assigned to the CH_3 and CH stretch bands, the 1196 cm^{-1} band was assigned to the stretching of C—O—C, and the 1093 cm^{-1} band was assigned to C—O stretching. After supercritical CO_2 processing, there was no change in the chemical composition of the samples; the results were consistent with the report by Kang [42]. These results indicate that the supercritical-CO_2-assisted phase inversion process is a physical process capable of producing PLLA scaffolds.

3.5. XRD and DSC analysis

To investigate the physical state of PLLA before and after the supercritical CO_2 processing, XRD analysis was performed. Fig. 6 shows the XRD spectra of the as-received PLLA and the PLLA scaffold. The main peaks at 16.3° and 18.5° proved the presence of

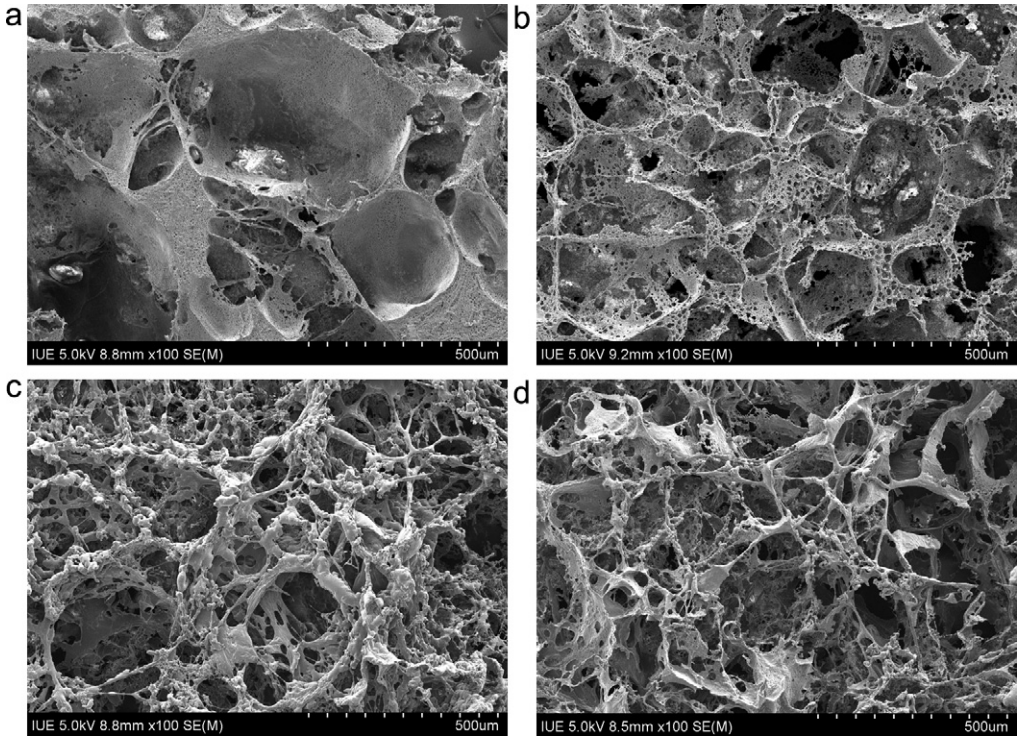

Fig. 4. SEM images of the scaffolds prepared from different AB/PLLA ratios: (a) 10:1, (b) 20:1, (c) 30:1, (d) 40:1.

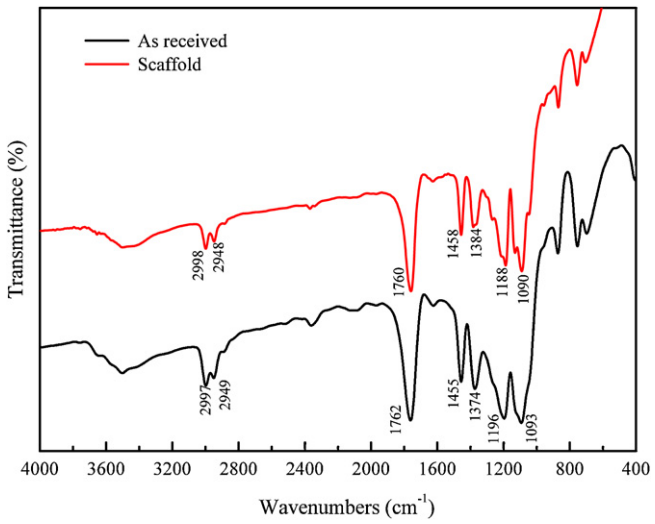

Fig. 5. FTIR spectra of as-received PLLA and the scaffold prepared by the supercritical fluid process.

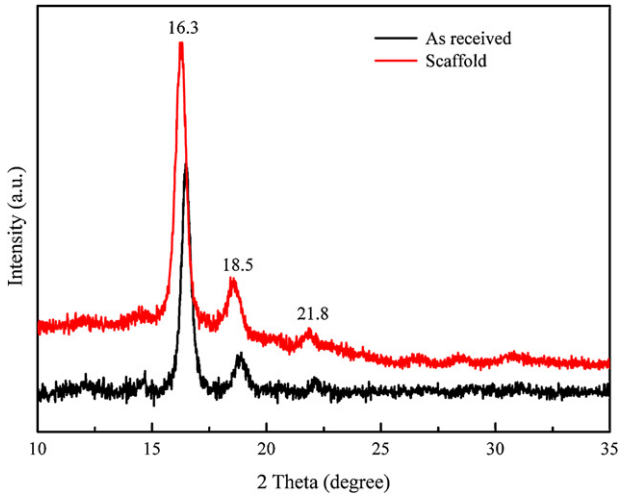

Fig. 6. XRD spectra of as-received PLLA and the scaffold prepared by the supercritical fluid process.

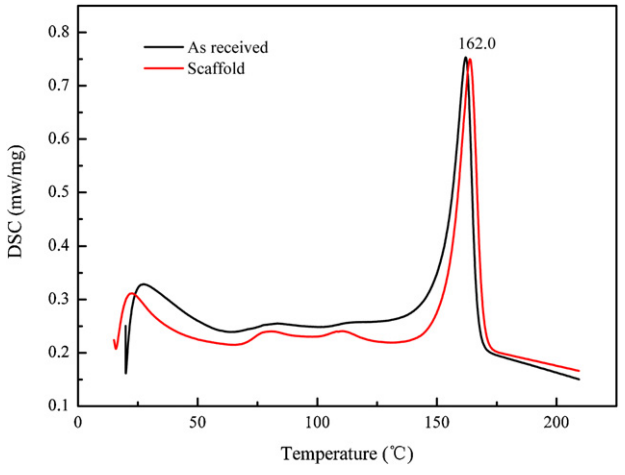

Fig. 7. DSC curves of as-received PLLA and the scaffold prepared by the supercritical fluid process.

semi-crystalline domains of PLLA specimens. Despite treating by the supercritical CO_2 process, XRD analysis showed that PLLA sample was still in semi-crystalline state. This result was similar to the previous study [43]. The DSC curves in Fig. 7 show that PLLA was observed as a semi-crystalline polymer with a melting temperature (T_m) of about 162.0 °C. The melting temperatures of the as-received PLLA and the scaffold are slightly shifted to a higher temperature, indicating that the supercritical-CO_2-assisted phase inversion process may not affect the crystallization of PLLA significantly [43,44].

4. Conclusion

Nanofibrous PLLA scaffolds with interconnected pores were successfully prepared using ammonium bicarbonate as a pore forming agent in a supercritical CO_2 process. The scaffolds possess the structural characteristics of large pores and micro and nanometer pores that have to be simultaneously present for tissue engineering applications. They are characterized by a high porosity (up to 97.5%), a fibrous structure that allows cell growth, a controllable cell size (depending on the particle size of AB), adequate mechanical properties, and almost no solvent residues. Moreover, it is possible to solve the problem of loss of the growth factors in the scaffold that occurs during the traditional preparation methods.

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