
The Fabrication and Characterization of Poly(lactic acid) Scaffolds for Tissue Engineering by Improved Solid–Liquid Phase Separation

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ABSTRACT

In this article a new technique was developed to fabricate scaffolds with a unique microstructure by solid–liquid separation in combination with particulate-leaching. Firstly, the effects of polymer concentration, quenching temperature on the porous morphology and the mechanical property of obtained scaffolds during solid–liquid separation have been investigated. Then, salt granules as porogen were introduced into the solid–liquid phase separation to produce the unique pore structure of the scaffold. The pore diameter of the scaffold could be controlled with the particulate size and the wall of pores possessed special microstructure, which enhanced the pore interconnectivity. The cell culture results confirmed that a good interconnectivity of the scaffold prepared by the improved solid–liquid separation was useful for nutrition transportation and cell proliferation. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: phase separation; microstructure; polyesters; scaffold; cell culture

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INTRODUCTION

Porous scaffolds composed of biodegradable polymers have been extensively explored in tissue engineering. Aliphatic polyesters, such as poly(lactic acid), are important kinds of biodegradable polymers approved by FDA for human clinical use [1–4]. These synthetic biodegradable polymers have been employed in many strategies in tissue engineering as scaffolds to direct specific cell growth and differentiation [5].

Ideally, a scaffold should have the following characteristics: (a) high porosity to provide adequate space for cell seeding or growth and flow transport of nutrients and metabolic waste; (b) biodegradable or bioresorbable with a controllable degradation and resorption rate to match cell/tissue growth *in vitro* and/or *in vivo*; (c) suitable surface chemistry for cell attachment, proliferation and differentiation; (d) mechanical properties to match those of the tissues at the site of implantation, and be easily processed to form a variety of shapes and sizes; (e) uniformly distributed and interconnective porous structure [6]. This is very important so that cells are easily distributed throughout the device and an organized network of tissue constituents can be formed. In order to meet the requirements of an ideal scaffold, several techniques have been developed: solvent casting/

salt-leaching [7], emulsion freeze-drying [8], gas-foaming [9], fibre-bonding [10], three-dimensional-printing [11] and particle sintering [12], etc. A thermally induced phase separation process has been used for tailoring a porous biodegradable polylactide foam. This method is easily implemented by freeze-drying a polylactide solution in a suitable solvent. Porous biodegradable polyurethane foams have been successfully prepared by freeze-drying for the reconstruction of meniscus lesions [13, 14]. Schugens *et al.* carefully investigated the influence of cooling rate on the morphology of scaffolds and found that the faster quenching resulted in a decrease of the average pore size [15]. Ma and Zhang demonstrated a novel method of fabricating a microtubular scaffold with a uniaxial temperature gradient by solid-liquid phase separation [16]. However, the pore diameters of these scaffolds (less than 100 μm) were too small to allow many kinds of cell transplant into the inner scaffold, because they were unsuitable pore sizes to accommodate the different cells to stretch and grow. It was reported that pore size should be close to 20 μm for the ingrowth of hepatocytes, 20–150 μm for skin regeneration, and in the range of 200–400 μm for bone regeneration [17]. The pore diameters and porosity of the scaffolds fabricated by solvent casting/salt-leaching could be easily controlled by the size and amount of particulates though there were very few close pores in these scaffolds as reported earlier in the literature [18].

In this study, we focused on the solid-liquid phase separation of semi-crystalline poly L-lactide in pure 1,4-dioxane because the scaffolds prepared by the phase separation method had high porosity and good mechanical properties. It was emphasized how the formulation and processing parameters of this porogen method influenced the pore morphology, the pore size, the foam mechanical property and the porosity. Special attention was paid to the effects of the polymer concentration, the quenching temperature. Unfortunately no matter how the condition changed, the pore diameters of the scaffold with enough mechanical properties to be utilized were around 100 μm , which did not satisfy the different cells to stretch and grow. Therefore we developed a technique of phase separation in combination with particulate-leaching, the pore diameters of the scaffolds obtained by the technique were around 200 μm and the interconnectivity of the scaffolds was better than that of scaffold by single phase separation. It was confirmed that the pore structure was convenient for cells to attach and proliferate by cell culture results.

EXPERIMENTAL

Materials

The synthesis of poly(L-lactic acid) (PLLA) used in the present study has been described earlier in the

literature [19]. The PLLA was purified by dissolution of the polymer in chloroform and precipitation in ethanol, followed by drying in vacuum at room temperature for 48 hr. The PLLA ($M_w = 15,050$, $M_n = 12,120$, $M_w/M_n = 1.24$) was measured by Waters510 gel permeation chromatography equipped with Shodex KF-800 columns. 1,4-Dioxane, chloroform and other reagents were of analytical quality.

Scaffold Preparation

Scaffold preparation by solid-liquid phase separation: a known amount of polylactide was dissolved in dioxane to form solutions with desired concentrations. Four different concentrations of PLLA solution (0.5, 1.0, 3.0 and 5.0% w/v) were frozen at -5°C and maintained over 2 hr to induce solid-liquid phase separation completely. The clear 5% PLLA solutions were poured into aluminum foil molds ($4 \times 4 \text{ cm}^2$) which were fast-frozen in liquid nitrogen, ice-water bath (0°C) or freezer (-25°C), respectively, and then were maintained at those temperatures for over 2 hr to induce solid-liquid phase separation completely. Solvent was removed by freeze-drying for 3 days and the dried scaffolds were then kept in a desiccator until characterization or usage.

Scaffold preparation by improved solid-liquid separation: different amounts of sieved NaCl powders (200–250 μm) were added into 5% (w/v) PLLA solution, then these slurries were maintained at 0°C for over 2 hr to induce the solid-liquid phase separation completely. After the solvent was removed by freeze-drying for 3 days, the matrix was put into distilled water to leach out NaCl every 3 hr until no precipitation occurred when some drops of AgNO_3 solution were added. The dried scaffolds were then kept in a desiccator until characterization or usage. The weight ratio of PLLA/salt was varied at 1/22, 1/28, 1/34 and 1/40 to optimize the best ratio at which the porosity of scaffold can be matched with that of the scaffold prepared by the solid-liquid phase separation.

Scaffold Characterization

Scanning electron microscopy (SEM, HitachiS-530) was used to observe the internal pore morphology of the scaffolds, which were coated with gold using a sputter coater (Desk-II; Denton Vacuum Inc.). The compressive mechanical properties of the scaffolds were measured by dynamical mechanical analyzer (DMA, Perkin-Elmer). Each circular specimen with a diameter of 1 cm and a thickness of 3 mm was compressed with a balance of $2 \times 10^4 \text{ Pa}$. The compressive modulus was averaged from four measurements per sample, and expressed as mean \pm standard deviation (SD).

The porosity of the scaffold was measured by using a previous method [20]. In this improved method, a density bottle instead of a graduated cylinder was used to measure the density and the

porosity of the scaffold. Ethanol with density ρ_e was used as the displacement liquid and operated at 30°C. A density bottle filled with ethanol was weighed (W_1). A scaffold sample of weight W_s was immersed into the density bottle, and then was weighed (W_2). The scaffold saturated with ethanol was taken out of the density bottle and then the density bottle was weighed (W_3).

The following parameters of a scaffold including the volume of the scaffold pore (V_p), the volume of the scaffold skeleton (V_s), the density (ρ_s) and the porosity (ε) were calculated.

$$V_p = (W_2 - W_3 - W_s)/\rho_e$$

$$V_s = (W_1 - W_2 + W_s)/\rho_e$$

$$\rho_s = W_s/V_s = W_s\rho_e/(W_1 - W_2 + W_s)$$

$$\varepsilon = V_p/(V_p + V_s) = (W_2 - W_3 - W_s)/(W_1 - W_3)$$

Cell Seeding and Culture

Mouse 3T3 fibroblast cells were grown in 50-ml cell culture flask with Dulbecco's Modified Eagles Medium (Gibco) supplemented with 15% calf serum (Gibco) and 100 U/cm³ each of penicillin and streptomycin. Cell culture was maintained in a 37°C gas-jacked incubator equilibrated with 5% CO₂.

Two kinds of the porous PLLA scaffolds were prepared by solid-liquid phase separation alone and combined with the salt-leaching method. The porous scaffolds were cut into disks with a diameter of 7 mm and a thickness of 1.5 mm. Two groups of disks were pretreated by NH₃ plasma treatment and transferred to ultra-cleaning workstation for ultraviolet lighting for 2 hr to sterilize and then located in a 96 well tissue culture plate. A cell suspension (100 μ l) with a cell density of 3–5 \times 10⁵ cells/disk was seeded evenly into the disks with a micro-pipette. The cell-seeded disks were maintained at 37°C under 5% CO₂ for 3 hr and then 2 ml of culture medium was added to the wells. After the cells were cultured for 13 hr, the cell seeding efficiency was determined by counting the number of cells remaining in the wells. After the cells were cultured for 2, 4 or 6 days, the viability and proliferation of fibroblast cells was determinate by MTT (Thiazolyl Blue Colorimetry) assay. The disks were rinsed using PBS (phosphate buffer solution) three times and transferred to another 96 well plate. Then 2 ml of culture medium was added to each well. A 5 μ l of MTT solution (5 mg/ml) was freshly added to the culture well incubated at 37°C and 5% CO₂ for 4 hr. The upper medium was removed carefully and the intracellular formazan was solubilized by adding 2 ml of 0.04 mol/l HCl/iso-propanol to each well. The absorbance of the produced formazan was measured at 492 nm using a spectrophotometer (Shanghai Precision & Scientific Instrument Co. Ltd.). The data were averaged from five measurements per sample and expressed as mean \pm SD. The statistical significance between two sets of data was

calculated using the Student's *t*-test. Data were taken to be significant, when a *p*-value of 0.05 or less was obtained (showing a 95% confidence limit). In the hematoxylin and eosin (H&E) coloration method, two kinds of samples prepared by solid-liquid phase separation alone and combined with salt-leaching method were fixed by 10% formalin for 24 hr at 4°C after being cultured for 2 weeks and 4 weeks. Subsequently, the specimens were dehydrated in the graded ethanol series and embedded in paraffin. The samples were cut into slices (1 μ m) using microtome and then were washed by xylene to eliminate paraffin. After being stained by standard H&E, the slices were observed under an invert-microscope (Olympus IMT-2, A10PL) and photographs were taken.

RESULTS AND DISCUSSION

Solid-Liquid Phase Separation

The aim of this study was to get a three-dimensional scaffold with uniformly distributed and interconnective pore structure as well as with good mechanical properties. To achieve this aim, porous PLLA scaffolds were fabricated by solid-liquid separation and the effect of processing parameters on pore structure of the scaffolds was studied. Therefore, several PLLA porous scaffolds were obtained with different concentrations of PLLA solutions by the solid-liquid phase separation at -5°C. Figure 1 showed that the pore structure was more regular in foams prepared from PLLA solutions of 1.0% (w/v), 3.0% (w/v) and 5.0% (w/v) (Fig. 1B, 1C and 1D) than 0.5% (w/v) (Fig. 1A). The mechanical property of the scaffold produced with 0.5% (w/v) PLLA solution was so poor that it could not stand enough pressure to measure its porosity. The higher concentration solution of 1% (w/v) PLLA provided porous supports with regular polygon pore and thin pore walls (Fig. 1B), the compressive modulus of which was about 1 MPa (Fig. 2). Obviously the scaffold prepared by 1% (w/v) PLLA solution was too weak to be used in practice. The scaffolds prepared from 3% (w/v) and 5% (w/v) PLLA solutions not only showed a more uniformly porous structure but also have good mechanical properties (such as about 2.0 MPa and 4.0 MPa in modulus shown in Fig. 2). It was thought that the scaffolds prepared by 3% (w/v) and 5% (w/v) of PLLA solutions were appropriate to be utilized in tissue engineering.

However, Fig. 2 showed the compressive modulus of the scaffolds obtained at different quenching temperature. As the same polymer concentration used, the compressive modulus of PLLA scaffolds prepared at the different quenching temperatures was almost the same without statistical significance (*p* > 0.3 for each group of data of samples prepared at the different quenching temperatures from the same concentration polymer solution). It was considered that the quenching

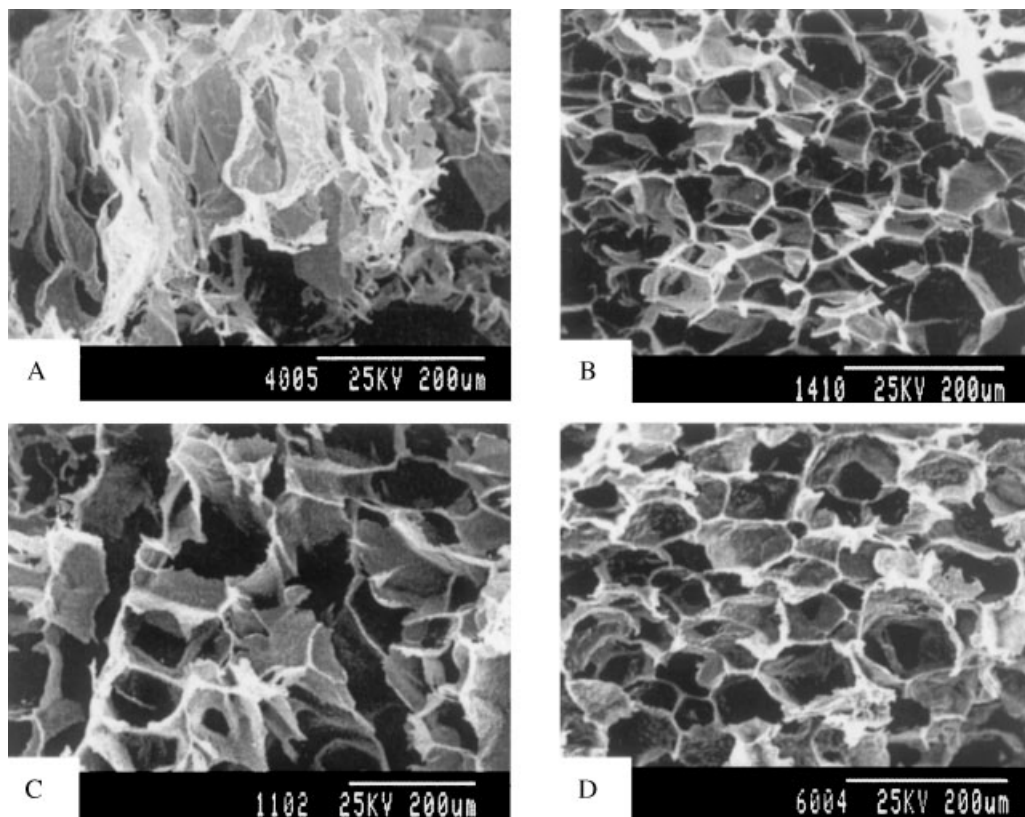


FIGURE 1. SEM pictures of cross-sections of PLLA scaffolds produced from different concentrations at -5°C : (A) 0.5%; (B) 1.0%; (C) 3.0%; (D) 5.0% (w/v).

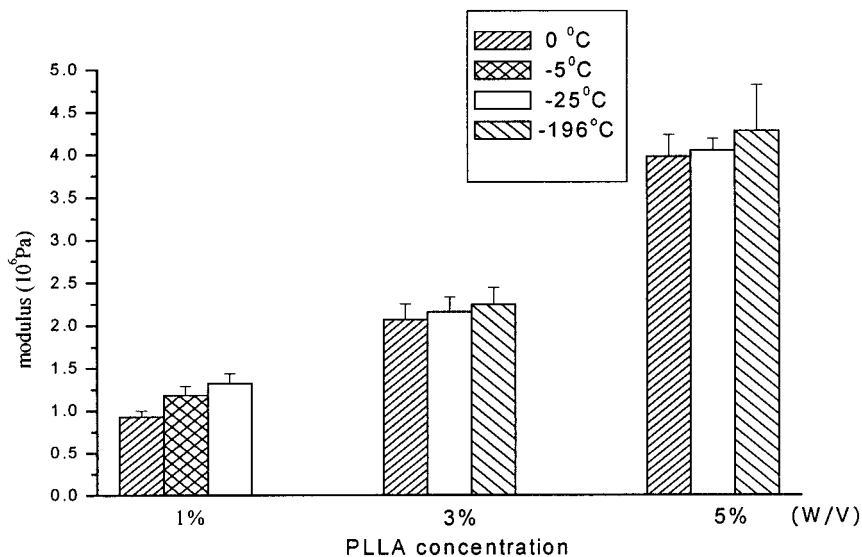


FIGURE 2. The compressive modulus of porous PLLA scaffolds prepared from different concentrations by solid-liquid phase separation at various quenching temperatures.

temperature was likely to make little contribution to the mechanical property of the scaffold and the polymer concentration played an important role in the mechanical property.

The effect of quenching temperature on the morphology of the scaffold obtained by 5% (w/v) was inspected as shown in Fig. 3. At -196°C , the SEM picture of the cross-section (Fig. 3E and 3F) showed a ladder-like structure, which was aniso-

tropic. The high magnification, Fig. 3E, illustrated the pore between two channels was $20\ \mu\text{m}$ or so. The cross-section picture of a foam produced at -25°C (Fig. 3C and 3D) showed a general pattern of lamellae with a main radial orientation from a central pore, the diameter of which was about $200\ \mu\text{m}$. Some inter-lamellar regions were subdivided into pores with pore diameter around $20\ \mu\text{m}$. The cross-section picture of scaffold prepared at

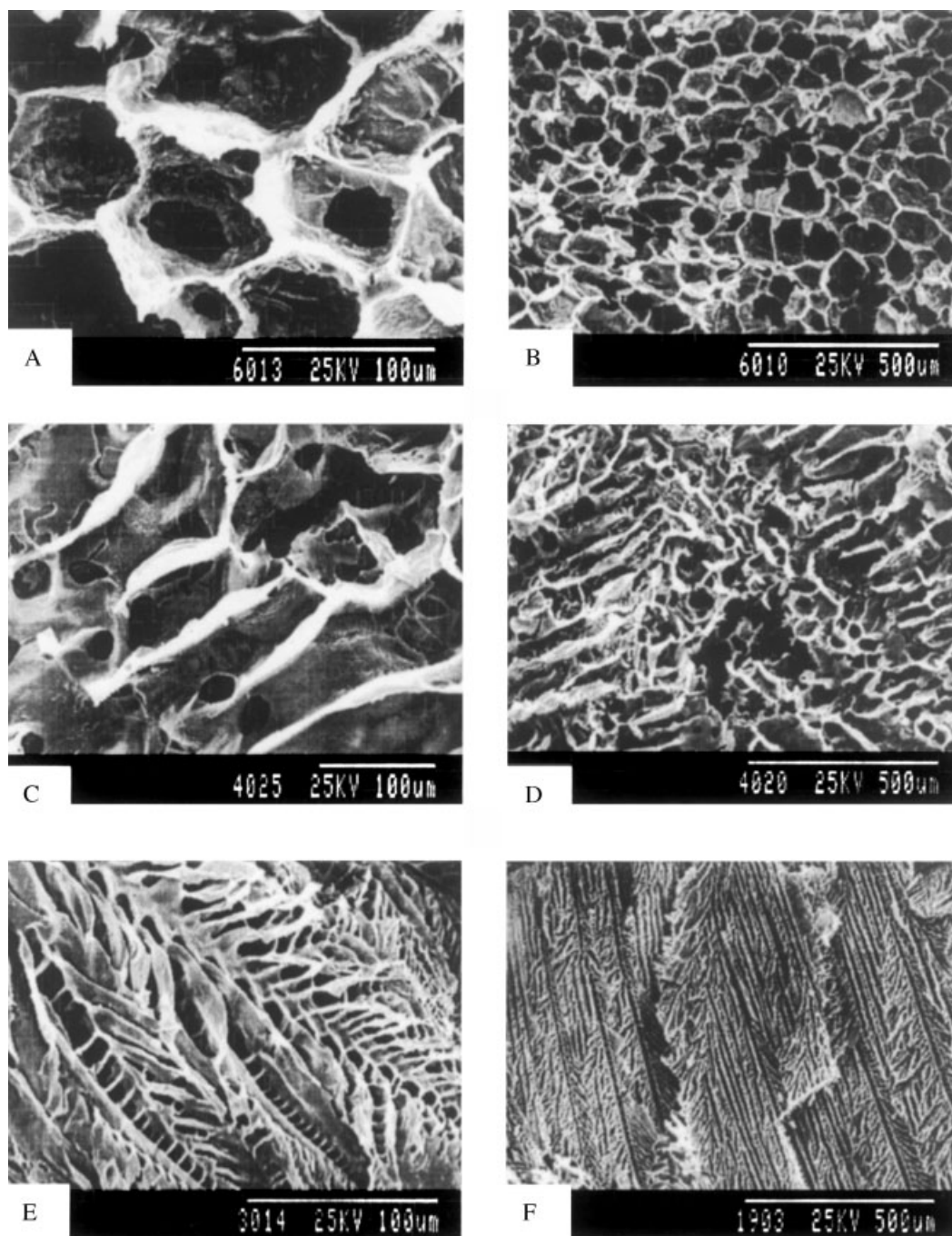


FIGURE 3. SEM picture of the cross-sections of scaffolds produced from 5.0% (w/v) PLLA solution at different quenching temperatures of 0°C (A and B), -25°C (C and D) and -196°C (E and F). Low magnification: B, D and F; high magnification: A, C and E.

0°C (Fig. 3A and 3B) demonstrated a regularly oval porous structure with pore diameter less than 100 μm, presenting an obvious isotropy. It was found that the morphologies of the scaffolds varied not only from ladder-like structure to regular pore structure but also from ladder-like structure to regular pore structure but also from anisotropy to isotropy as the quenching temperature was increasing from -196 to 0°C. It was assumed that the pore structure of the scaffold in the solid-liquid phase separation was controlled by the crystals of dioxane [21]. It was also noticed that the pores in the scaffold

produced from 5% (w/v) PLLA solution at -196°C were the smallest, which was about 20 μm, but the pores in the scaffold produced at 0°C or -25°C were much larger. The reason of this phenomenon was that the high quenching temperature was favored to obtain large crystals of dioxane leading to large pores in the scaffold.

From the data listed in Table 1, it was shown that the change of quenching temperature had little influence on the porosity of scaffolds, in other words, the porosities of scaffolds produced

TABLE 1. The Characterization of PLLA Scaffolds Produced from Various Concentrations at Different Temperatures

Polymer (w/v)	Temperature (°C)	Macroscopic aspect	Porosity (%)
5.0	0	Rigid foam	91.3
5.0	-25	Rigid foam	91.4
5.0	-196	Rigid foam	91.6
3.0	0	Soft foam	96.4
3.0	-25	Soft foam	96.1
3.0	-196	Soft foam	96.1
1.0	0	Soft foam	98.0
1.0	-25	Soft foam	98.4
1.0	-196	Soft foam	—
0.5	-5	Cotton wool	—

with the same concentration of PLLA solution at different temperatures were almost the same, because the pore formation in the scaffold was caused by the crystallizing and lyophilizing the solvent in PLLA solution. The lower the polymer concentration was, the more crystallization of the solvent formed and the higher the porosity of the scaffold. The porosity of the scaffolds increased only when the concentration of PLLA solution decreased.

Improved Solid-Liquid Separation

Based on the results of experiments in the solid-liquid separation mentioned earlier, it was found that the scaffolds produced by the solid-liquid separation had good interconnectivity but the pore diameters of the scaffolds were so small (usually less than 100 μm) that these scaffolds would hardly meet the requirements for osteoblast, fibroblast cell culture and skin regeneration in tissue engineering. In the previous research, the pore diameters of the scaffold produced by solvent casting/salt-leaching method were controlled by the particulate size from 100 to 300 μm , however few close pore structures in the scaffolds were observed in the SEM picture [22]. Therefore

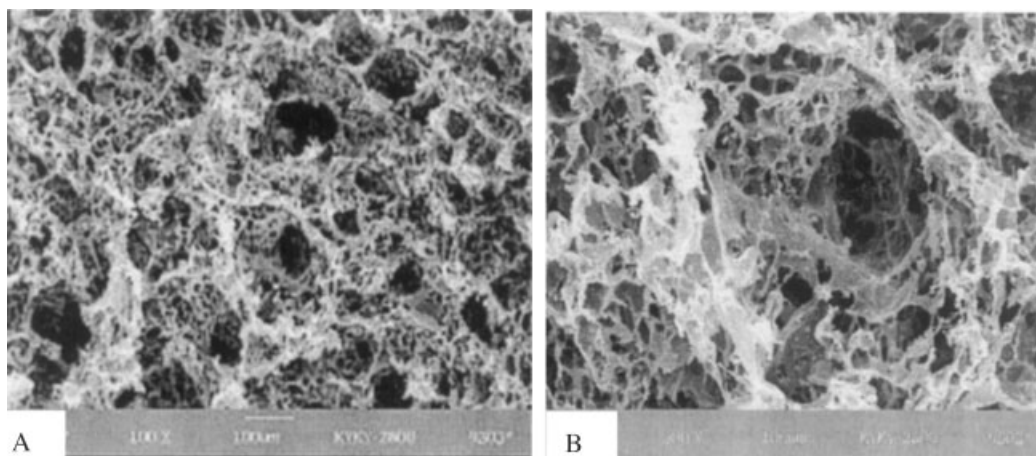
the solid-liquid separation combined with the salt-leaching method was developed to obtain the interconnective scaffold, where the pore size could be controlled to meet the cell culture requirements of the different types of cells, such as skin fibroblast cells and osteoblast.

The four different amounts of salt were added into the 5% PLLA solution evenly and frozen at 0°C for over 2 hr, then the phase-separated polymer/solvent/salt systems were freeze-dried under vacuum for 3 days. The cross-section SEM picture of the scaffold produced by the improved solid-liquid phase separation at the weight ratio of 1/34 (polymer/salt) is shown in Fig. 4. It was clear that there was a very unique pore structure in the scaffold in which some distinct pores around 10 μm were interspersed in the wall of many big pores with a mean diameter of about 200 μm . The small pores left behind by the crystals of the solvent after freeze-drying improved the pore interconnectivity of the scaffolds. The data in Table 2 demonstrated that the scaffolds of high porosity had good compressive modulus (more than 2 MPa). The porosity and the mechanical properties of the scaffolds could be adjusted by adding different amounts of salt. The big pore diameter of the scaffold could be controlled by changing the particulate size. In fact the fabrication technology described here was versatile and had general applicability to other polymers.

TABLE 2. The Characterization of PLLA Scaffolds Prepared by Improved Solid-Liquid Separation Method at 0°C

PLLA/salt (w/w) ^a	Porosity (%)	Modulus (MPa)
1/22	93.5	3.6
1/28	95.5	3.1
1/34	96.4	2.3
1/40	98.5	2.1

^a The used salt particulate size was 40–60 mesh.

**FIGURE 4.** SEM picture of the cross-sections of scaffolds with the weight ratio of 1/34 (PLLA/NaCl) by improved solid-liquid phase separation at 0°C. (A) $\times 100$, (B) $\times 300$.

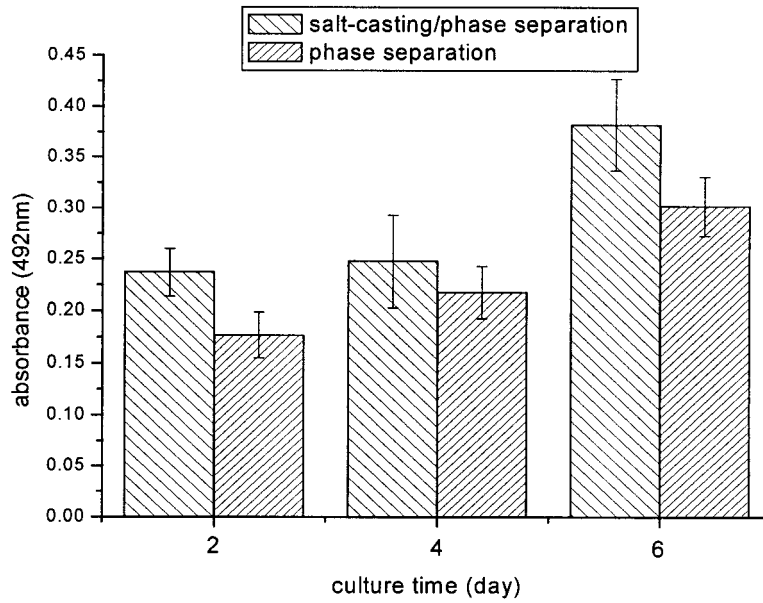


FIGURE 5. Comparison of cell growth on two different kinds of scaffolds for 2, 4 and 6 days ($p < 0.1$).

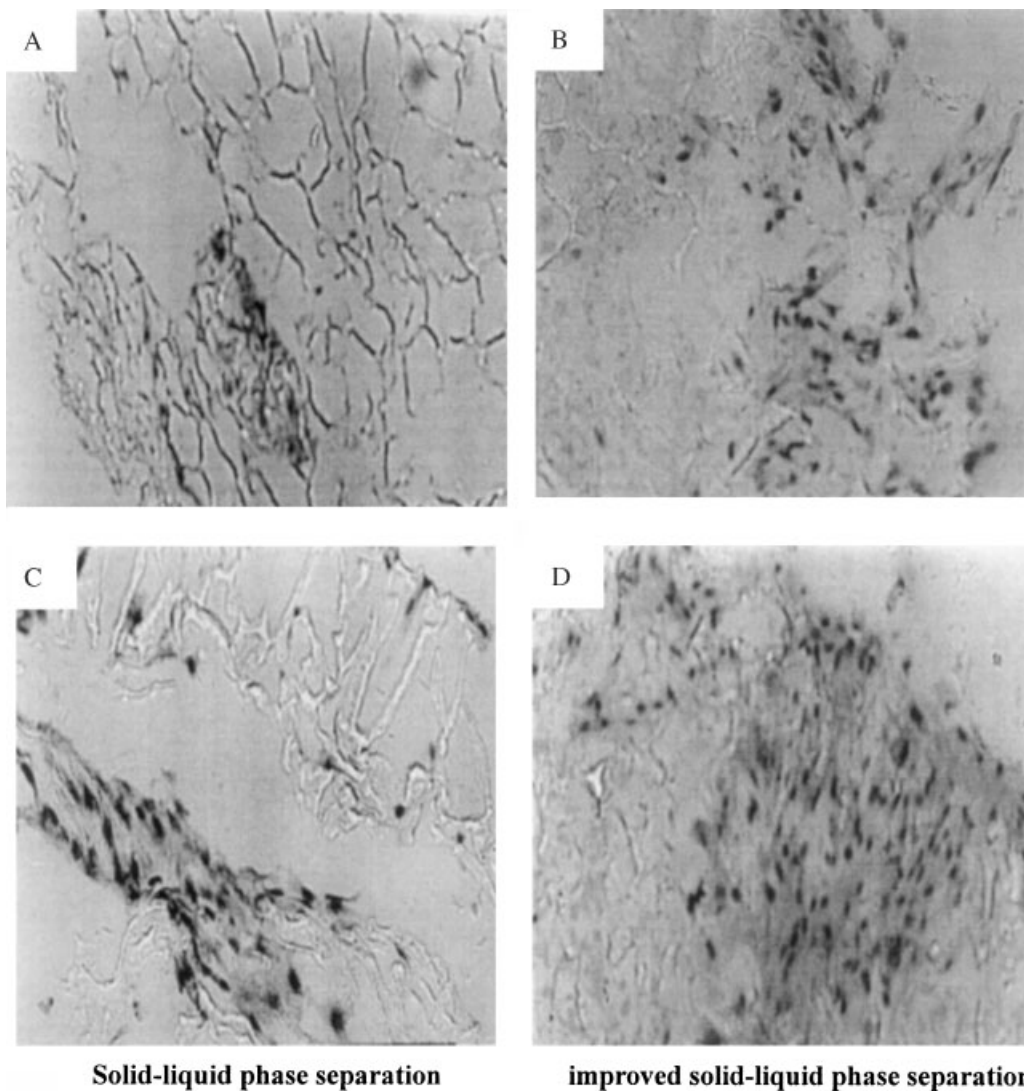


FIGURE 6. H&E picture of fibroblast cells in PLLA scaffolds by two different methods. (A) and (C) Cultured for 2 weeks, (B) and (D) cultured for 4 weeks.

Cell Culture on Two Kinds of Scaffold

The effect of special pore structure of the scaffold on cell's proliferation and growth was evaluated and compared with the scaffold prepared by single solid-liquid separation method. Therefore, MC3T3 fibroblast cells were seeded on the two kinds of scaffolds possessing the approximate porosity (96%). The two kinds of PLLA scaffolds were modified by NH₃ plasma treatment before cell seeding in order to insure that the amounts of cells seeded in the scaffolds were equal [23]. The MTT assay (Fig. 5) indicated that the higher absorbance was obtained in the scaffold prepared by the improved solid-liquid separation whenever the cells were cultured in 2, 4 or 6 days. It meant that the viability of cells in the scaffold prepared by improved phase separation was better. After cultured for 2 and 4 weeks, the cells in the scaffolds prepared by improved phase separation (Fig. 6B and 6D) were more viable than the cells in the scaffolds prepared by solid-liquid phase separation (Fig. 6A and 6C). Therefore it was demonstrated that the interconnectivity would be good to facilitate the exchange of nourishments and oxygen, which was beneficial to the cell's growth and proliferation.

CONCLUSION

It had been shown that various microcellular and porous scaffold morphologies could be obtained by adjusting the parameters during solid-liquid phase separation. The polymer concentration played a vital effect on the porosity as well as on the mechanical property of the scaffold, and the pore morphology of the scaffold could be controlled by the quenching temperature. A new protocol was also developed to combine solid-liquid separation with the salt-leaching method. The pore diameter of the scaffold by the new technique could be adjusted to satisfy the various cell growth for tissue engineering and the wall of pores in scaffolds possessed special microstructure causing better interconnectivity. It was proven that the good interconnectivity would facilitate the delivery of nutrient, oxygen and metabolites, which was beneficial to cell's proliferation and differentiation. It was also demonstrated that the more interconnectivity the scaffold was, the more vigorously the cells would propagate.

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