Preparation and Characterization of Poly(L-lactide)/ Poly(ϵ-caprolactone) Fibrous Scaffolds for Cartilage Tissue Engineering

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ABSTRACT: Polyblend fibrous scaffolds in mass ratios of 100/0, 90/10, 80/20, and 70/30 from poly(L-lactide) (PLLA) and poly(ϵ-caprolactone) (PCL) for cartilage tissue engineering were prepared in three steps: gelation, solvent exchanging, and freeze-drying. Effects of the blend ratio, the exchange medium, and the operating temperature on the morphology of scaffolds were investigated by SEM. PLLA/PCL scaffolds presented an ultrafine fibrous network with the addition of a "small block" structure. Smooth and regular fibrous networks were formed when ethanol was used as the exchange medium. Properties of the scaffolds, such as thermal and mechanical properties, were also studied. The results suggested that the compressive modulus declined as PCL amount increased. The incorporation of PCL effectively contributed to reduce the rigidity of PLLA. Bovine chondrocytes were seeded onto PLLA/PCL scaffold. Cells attached onto the fibrous network and their morphology was satisfactory. This polyblend fibrous scaffold will be a potential scaffold for cartilage tissue engineering. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 91: 1676–1684, 2004

Key words: matrix; fibrous scaffolds; tissue engineering; gelation; blends

INTRODUCTION

An artificial extracellular matrix—that is, a scaffold—is always needed in tissue engineering temporarily to repair or regenerate the defective or lesion tissues. The porous scaffold serves as a three-dimensional template for initial cell attachment and subsequent tissue formation both *in vitro* or *in vivo*. Cells can be expanded in culture and seeded onto a scaffold that will slowly degrade and be absorbed as the tissue structures grow. In the past, several materials, either from biological original (collagen,[1] gelatin[2] or/and chitosan,[3,4] and hyaluronic acid[5] and derivative, etc.) or synthetic materials (polyglycolide PGA,[6] optically active and racemic polylactides, PLLA, PDLLA,[7] polycaprolactone PCL,[8] etc.), have been tried for this purpose with more or less success. It is well known that the architecture and mechanical properties of the scaffolds have decisive effects on the regenerating tissue. The morphology of the scaffolds can be expected to significantly affect cell seeding and mass transport within the three-dimensional matrix.[9] There have been several methods to fabricate highly porous polymer scaffolds. Mikos and coworkers[10] prepared PLLA foam through particulate-leaching employing salt as the porogen. Nam et al.[11] prepared PLLA foam through combining the method of gas foaming and particulate leaching that enable PLLA paste to be molded into any shape, allowing for fabricating a wide range of tissue scaffolds. Phase separation is the most common way to produce PLLA foam. Pore morphology of the foam can be controlled by changing several parameters during the process, such as polymer concentration, solvent/nonsolvent composition, quenching route, and quenching depth.[12–16] Ma and Zhang[17] developed a new technique that combined thermally induced gelation and freeze-drying to mimic natural extracellular matrix architecture. The PLLA scaffold was composed of ultrafine fibrous network. Phase-separation conditions also affected the morphology of the fiber. The advantages of synthetic polymers with extracellular matrixlike architecture rendered this scaffold potentially useful for applications in tissue engineering.

PLLA is about 37% crystalline with a melting point of 175–178°C and a glass-transition temperature (T_g) of 60–65°C, whereas poly(ϵ-caprolactone) (PCL) is a semicrystalline polymer with a melting point of 59–64°C and a T_g of −60°C. PLLA's limitation in application, attributed to its brittleness, can be improved by copolymerization or polyblending with PCL. Either physical blending or copolymerization provides the optimal mechanical properties to the materials.[18–21] Meck et al.[8] used an amorphous copolymer of DL-

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lactide and ϵ-caprolactone as a biodegradable nerve guide. However, investigations are lacking on preparing tissue-engineered scaffolds composed of PLLA/PCL polyblend.

In this study, the PLLA/PCL polyblend ultrafine fibrous scaffolds with different compositions were fabricated by gelation and freeze-drying. The effect of addition of PCL on morphology of the scaffolds was investigated. The thermal and mechanical properties of PLLA/PCL scaffolds were described. The fibrous network scaffolds reported hold promise for tissue regeneration.

EXPERIMENTAL

Materials

PLLA was supplied by Zhejiang University (Hangzhou, China). It was purified by precipitation of its chloroform solution in ethanol. The PLLA was air-dried and vacuum-dried before use. PCL and poly(L-lactide)-copoly(ϵ-caprolactone) in 2 : 1 monomer ratio (PLLA-*co*-PCL) were from Institute of Chinese Applied Chemistry Academy of sciences (Changchun) and used without further purification. Values of intrinsic viscosity [η] of PLLA, PCL, and PLLA-*co*-PCL, measured by the viscosity method in a dilute polymer/chloroform solution (0.1 g/dL) using a Ubbelohde viscometer (capillary diameter 0.47 mm; Cannon–Ubbelohde, State College, PA) at 25°C, were 3.079, 3.777, and 2.775 dL/g, respectively. All other chemicals were analytical grade.

Preparation of PLLA/PCL fibrous scaffolds

Certain amounts of PLLA and PCL with PLLA/PCL mass ratios of 100/0, 90/10, 80/20, and 70/30, respectively, were dissolved in tetrahydrofuran (THF) to form 2.5% (w/v) solutions. Clear solutions were obtained under magnetic stirring at about 50°C. The solution was poured into a custom-made mold composed of two stainless steel plates and a silicone sealed ring. The solution-containing mold was rapidly transferred to a refrigerator and stored at −18°C for 3 h for gelation. The gel was then immersed several times into distilled water (4°C) or ethanol (−18°C) to thoroughly exchange the THF. After solvent exchanging the gel's structure became spongelike. After being removed from the exchange medium, the sponge was blotted and placed in the refrigerator again at −18°C for at least 2 h. Then it was charged to a glass vessel immersed in an ice/salt bath at about −10 to −15°C. The fibrous scaffold was finally obtained by freeze-drying thoroughly at less than 5 Pa vacuum.

Morphology observation

The morphology of the PLLA/PCL scaffolds was viewed under a scanning electron microscope (Philips XL30; Philips, The Netherlands). The fracture sections of liquid nitrogen frozen specimens were coated with gold before observation.

^{1}H-NMR assay

^{1}H-NMR assay of PLLA/PCL specimens was carried out with a Varian Mercury_VX300 spectrometer (Varian Associates, Palo Alto, CA). $CDCl_3$ was used as the solvent and tetramethylsilane as an internal reference. ^{1}H-NMR peak areas were determined by spectrometer integration and reported as relative intensities representing a given number of protons in the macromolecules.

Thermal analysis

Thermal properties of the fibrous scaffolds were determined by a differential scanning calorimeter (DSC; Netzsch DSC 204, Germany) under a nitrogen gas flow. Specimens were quenched to −100°C and then heated to 220°C at a rate of 10°C/min. The melting temperatures of PLLA and PCL were noted as temperatures at the maximum values of their melting peaks, respectively. The melting enthalpies ($\Delta H_{m,\mathrm{PLLA}}$ and $\Delta H_{m,\mathrm{PCL}}$) were referred to as the corresponding energies (J/g) (the peak areas above the baseline) at the melting range. The crystallinities of PLLA ($X_{c,\mathrm{PLLA}}$) and PCL ($X_{c,\mathrm{PCL}}$) were determined by rating their melting enthalpies to 93 J/g (the melting enthalpy of 100% crystalline PLLA) and 142 J/g (the melting enthalpy of 100% crystalline PCL) by the following equations[22]:

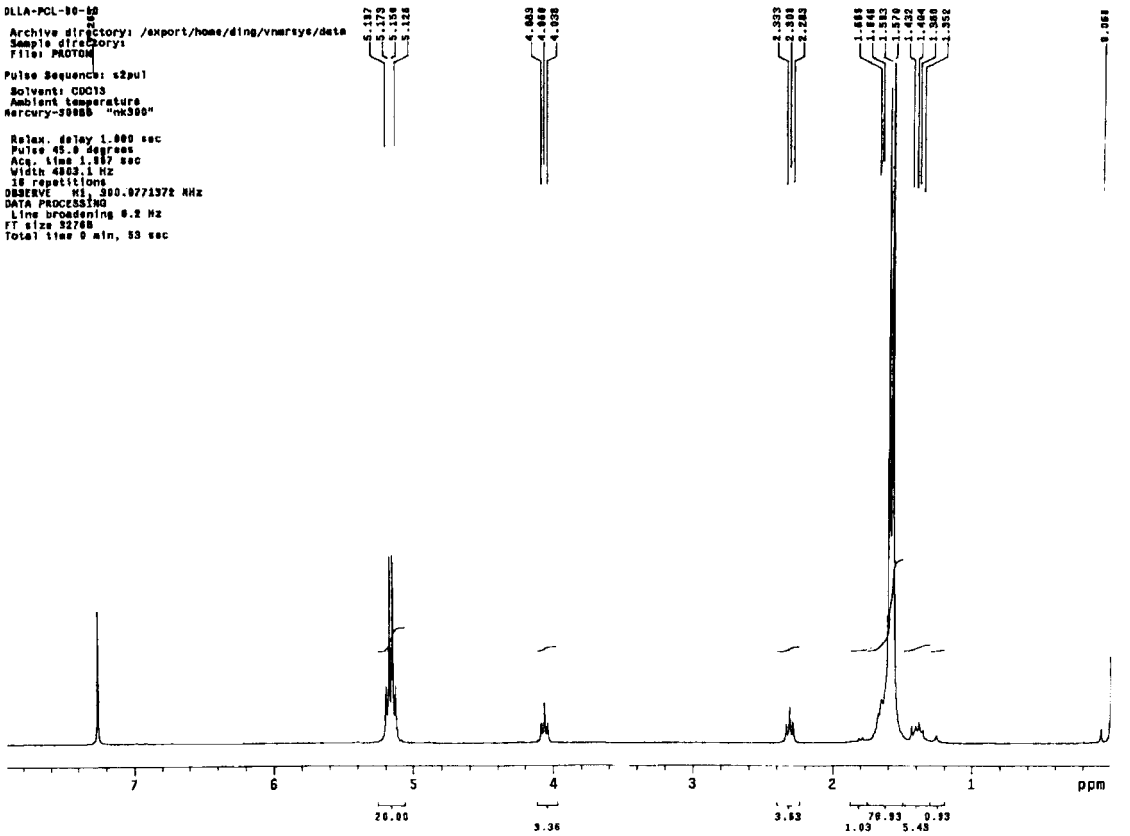
$$X_{c.\mathrm{PLLA}}(\%) = 100 \times \Delta H_{m.\mathrm{PLLA}}/(m_{\mathrm{PLLA}} \times 93) \quad (1)$$

$$X_{c.\mathrm{PCL}}(\%) = 100 \times \Delta H_{m.\mathrm{PCL}}/[(1 - m_{\mathrm{PLLA}}) \times 142] \quad (2)$$

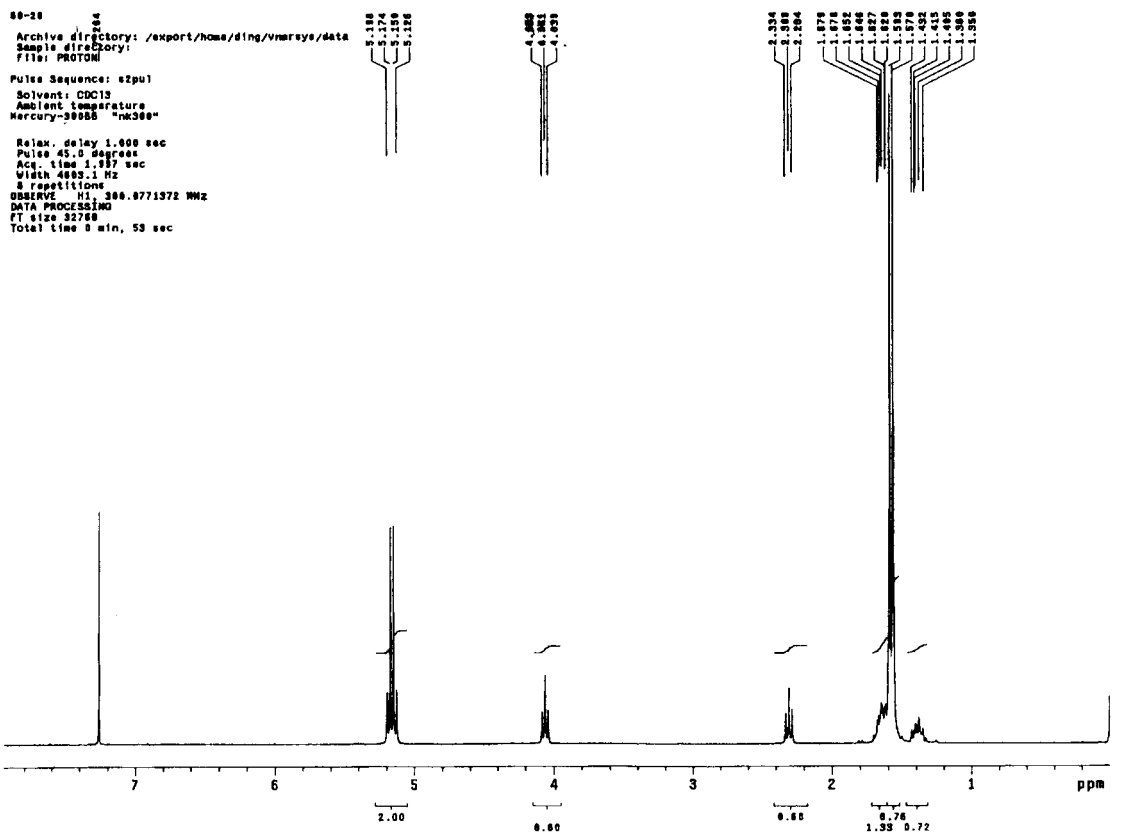
where m_{PLLA} represents PLLA mass percentage in the blended scaffolds.

Mechanical properties

The compressive mechanical testing was carried out in a M500–10AX Testometric universal tester (The Testometric Co. Ltd., UK) at 25°C with a 250-N load cell. The crosshead speed was 1 mm/min. Circular disks of the scaffold specimens with diameter of 6.0 mm and height of 5.5 mm were used. Scaffolds were tested at both dry and wet states. The scaffolds were soaked in phosphate-buffered saline (PBS) at 25°C for 48 h. The compressive modulus was evaluated from the initial region of stress–strain curves. At least five specimens were tested for each specimen. Also the averages and standard deviations were graphed.

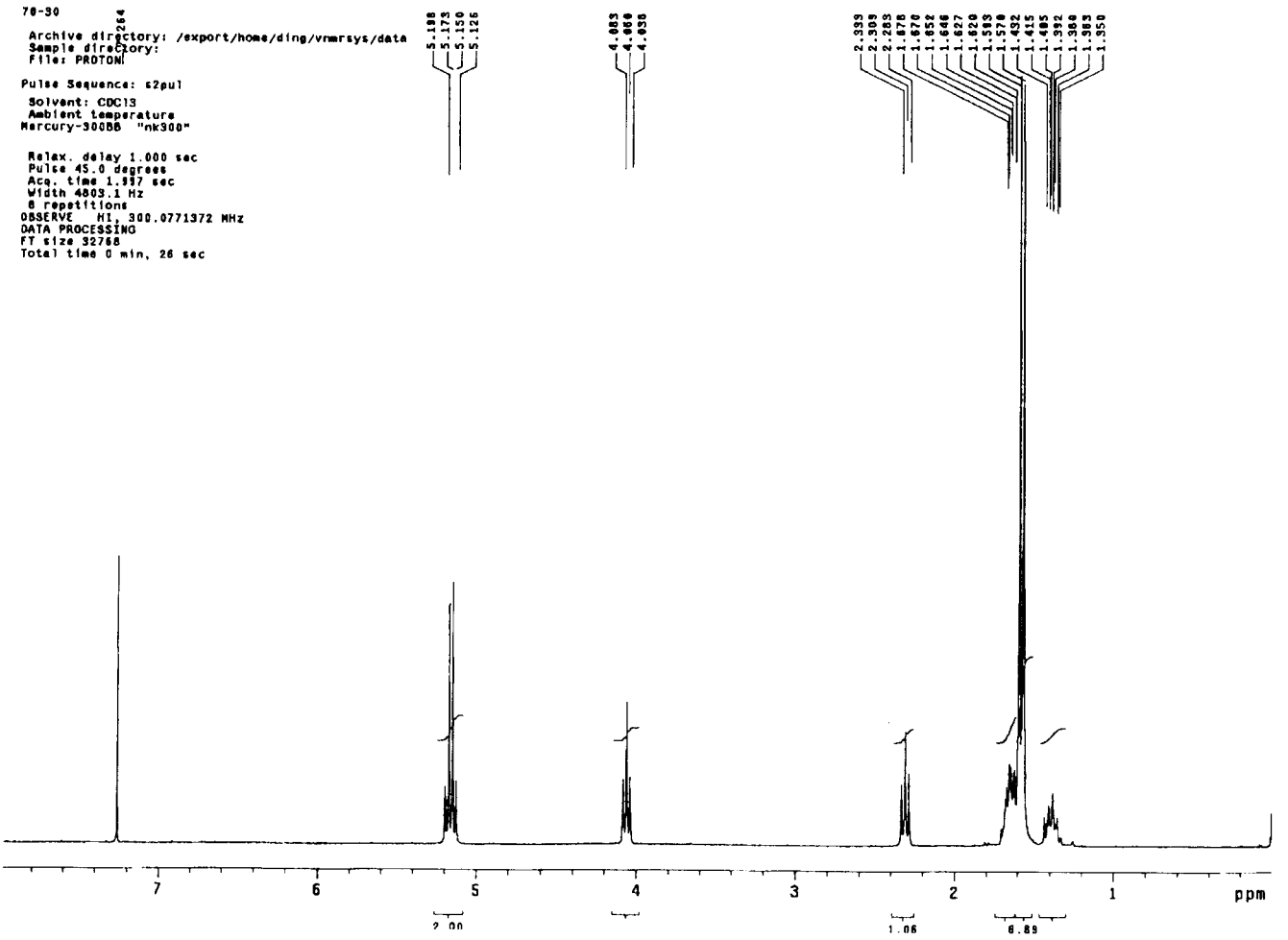


(a)



(b)

Figure 1 ^{1}H-NMR spectra of PLLA/PCL scaffolds with different compositions: (a) 90/10; (b) 80/20; (c) 70/30.



(c)

Figure 1 (Continued)

Cell culture on PLLA and PLLA/PCL scaffolds

Chondrocytes derived from bovine femoropatellar cartilage were cultured in cell culture flasks with Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 584 mg/L glutamine, 15% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, 10 mM *N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid (HEPES), 0.4 m*M* proline, and 50 μg/mL ascorbic acid. Cell culture was maintained in an incubator equilibrated with 5% CO_2 at 37°C. The medium was changed every 3 days. When the cells had grown to 80% confluence, they were digested by 0.25% trypsin from the culture flask and made into a cell suspension.

PLLA scaffold and PLLA/PCL (80/20) polyblend scaffold prepared as above were cut into small cubes (6 mm in length and 6 mm in width) with a stainless steel blade. All the specimens were irradiated under Co^{60} γ-ray (25 kGy) to be sterilized. After that, they were prewetted in Dulbecco's PBS, which contained 30% ethanol for 0.5 h, and then transferred into an excess amount of PBS for 24 h. After having been soaked in DMEM for 0.5 h, the specimens were placed into 24-well tissue-culture plates.

Chondrocyte suspension (100 μL) with a density of 2.0×10^7 cells/mL was seeded evenly into the wells with a pipette. After the cell-seeded scaffolds were incubated at 37°C under 5% CO_2 condition for 1 h, 2 mL of the incubation medium (DMEM with 10% fetal calf serum) was added to the wells. The medium was changed once every 2 days. The cell-seeded scaffolds were cultured for 3 days.

RESULTS AND DISCUSSION

^{1}H-NMR analysis

In the ^{1}H-NMR spectra (Fig. 1) of the polyblend scaffolds, peaks of PLLA (at $\sim$ 1.57, 5.126 ppm) and PCL [at $\sim$ 1.35, 1.62 ppm (partly overlapped with one peak from PLLA), 2.283, and 4.038 ppm] can clearly be detected, indicating that the fibrous scaffolds were composed of both PLLA and PCL. The actual compositions of these scaffolds were obtained from the ratio

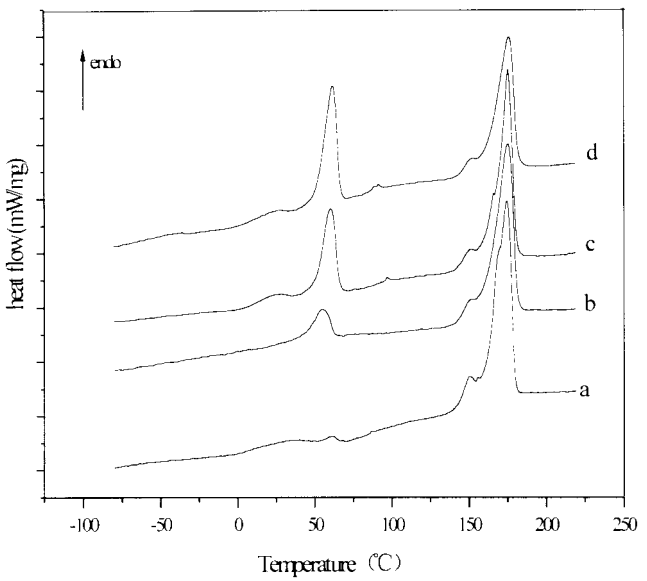


Figure 2 DSC curves of PLLA/PCL scaffolds: (a) 100/0; (b) 90/10; (c) 80/20; (d) 70/30.

of integration area of PLLA (δ_H = 5.126 ppm) to that of PCL (δ_H = 4.038 ppm). The real compositions were 88.26, 80.81, and 70.41 for the three polyblend scaffolds, 90/10, 80/20, and 70/30, respectively. It can be seen that the real compositions were far closer to the initial ones with which PLLA and PCL were fed at the beginning. Data revealed that the ratios of PLLA to PCL were not changed significantly during fabrication. The loss during solution blending and solvent exchanging could be negligible.

Thermal analysis

The DSC thermal curves of the scaffolds with different compositions are shown in Figure 2. There was no evident glass transition in the scans, probably because of the interference of PLLA and PCL crystallinities.

Using real compositions obtained from ^{1}H-NMR, the calculated crystallinities (X_c) of PLLA and PCL in polyblend scaffolds are listed in Table I. It was found that the melting point of PLLA ($T_{m,\mathrm{PLLA}}$) and $X_{c,\mathrm{PLLA}}$ remained nearly the same for m_{PLLA} = 100 with further increase of PCL amount in the scaffolds. This suggested that microscopic crystallization of PLLA was not strongly disturbed by the presence of PCL macromolecules. Thus, PLLA and PCL within the scaffolds were in a phase-separation state, at least to some degree. On the other hand, however, $T_{m,\mathrm{PCL}}$ exhibited a significant increase, whereas the crystallinity showed an obvious increase initially, followed by a subsequent reduction. This indicated that the crystallite growth of PCL was interrupted by the great amount of PLLA in spite of the excellent mobility of its own chain segments. Moreover, the nucleation of PCL crystallites was also disturbed by PLLA. Therefore, PLLA and PCL were not completely phase-separated. There might be some interaction between the PLLA and PCL macromolecules.

Morphology

PLLA/PCL polyblend scaffolds and PLLA-*co*-PCL scaffold were prepared through the same method of gelation, solvent exchanging, and freeze-drying. The morphology of the blend matrix exhibited an ultrafine fibrous network structure, whereas the copolymer scaffold had a porous structure with some "blocks" (Fig. 3). This result implied that the crystallization of the individual polymer played an important role in gel formation, as reported in the study by Ma and Zhang.[17] The copolymer of PLLA and PCL is amorphous in nature and cannot form a gel by crystallization, so the fibrous structure was not achieved.

Scaffolds of PLLA and PCL with different compositions using distilled water (4°C) as an exchange medium were all ultrafine fibrous network structure (Fig. 4). Quite different from the case of pure PLLA, when PLLA was blended with PCL, a fibrous network with the addition of "small block" structure was observed. These blocks formed because of bonds of adjacent fibers. With an increase of PCL amount, the volume of these "blocks" became increasingly larger. This phenomenon became most obvious for the composition of 70/30 of PLLA/PCL. Both PLLA and PCL were crystallizable and can form a gel at −18°C by crystallization. When quenching the polymer solution to −18°C, phase separation occurred by a spinodal decomposition mechanism. Then the solution was frozen further to turn into an ultrafine fibrous network gel. In the case of polyblending PLLA and PCL, however, there was phase separation between them to some extent as shown in DSC results. The rate of phase separation

TABLE I
Thermal Properties of PLLA/PCL Scaffolds

m_{PLLA} (%)	$T_{m,\mathrm{PLLA}}$ (°C)	$\Delta H_{m,\mathrm{PLLA}}$ (J/g)	$X_{c,\mathrm{PLLA,real}}$ (%)	$T_{m,\mathrm{PCL}}$ (°C)	$\Delta H_{m,\mathrm{PCL}}$ (J/g)	$X_{c,\mathrm{PCL,real}}$ (%)
100	174.8	61	66			
90	175.5	55	67	54.6	9	54
80	175.6	49	65	60.7	19	70
70	176.2	42	64	61.9	26	62

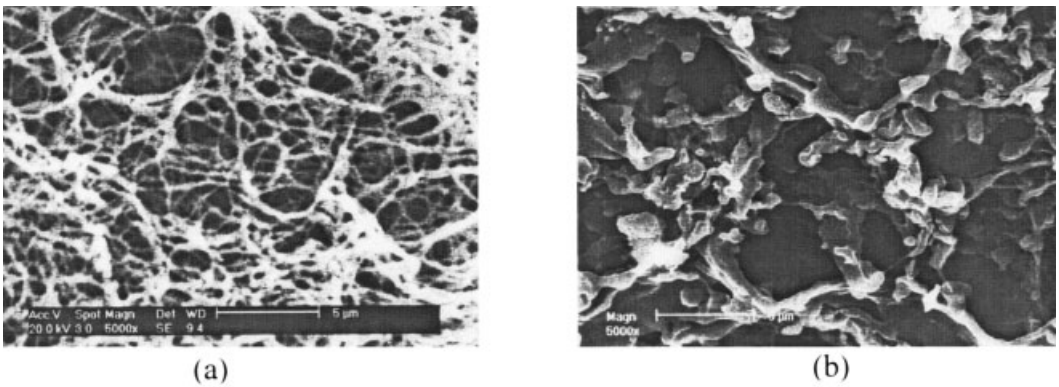


Figure 3 SEM micrographs of scaffolds prepared from 2.5 wt % [(a) PLLA/PCL (80/20)] and 5 wt % THF solution [(b) PLLA-*co*-PCL] at a gelation temperature of −18°C. (Original magnification ×5000.)

competed with that of gelation.[23] Therefore, coarsening of PCL microphases caused the "block" morphology within the scaffolds, given that the gelation time of PCL (60 min) was far slower than that of PLLA (22 min).

Morphologies of scaffolds using distilled water at different temperature were evidently unalike (Fig. 5). At 20°C, only a few fibers remained in the blended scaffolds, whereas there was no significant change in morphology of PLLA scaffolds. That is to say this result was mainly attributed to the damage of the gelation of PCL, which has excellent mobility of chain segments. A higher temperature, such as 20°C, promoted the movement of molecular chain segments and brought this result.

To reduce the damage of the gel, dehydrated ethanol was used as the exchange medium. Ethanol is a nonsolvent of PLLA and PCL and it will not solidify at −18°C. This enables the exchanging process to proceed at a lower temperature. The morphology of scaffolds at −18°C immersed in ethanol is shown in Figure 6. It was found that no evident "block" structures existed even in the matrix of the composition of 70/30 of PLLA/PCL. This indicated that the gel network structure was maintained at the lower temperature. Moreover, the surface of the fibers in Figure 6 was

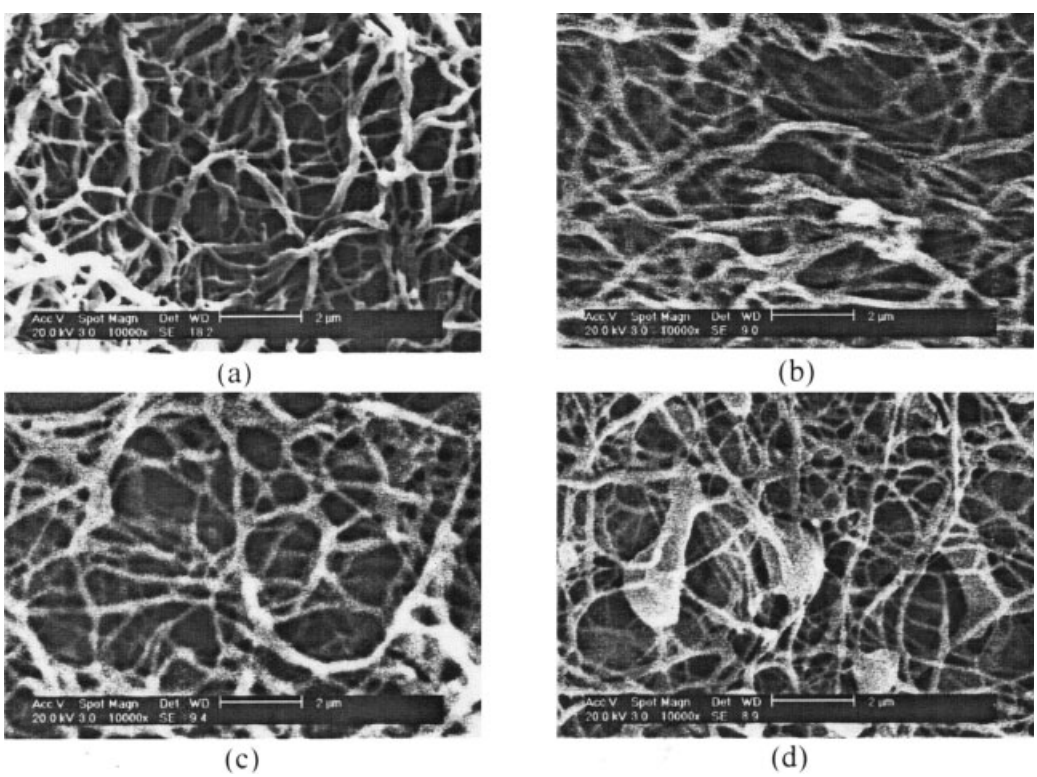


Figure 4 SEM micrographs of PLLA/PCL scaffolds with different compositions using distilled water as the exchanging medium at 4°C: (a) 100/0; (b) 90/10; (c) 80/20; (d) 70/30. (Original magnification ×10,000.)

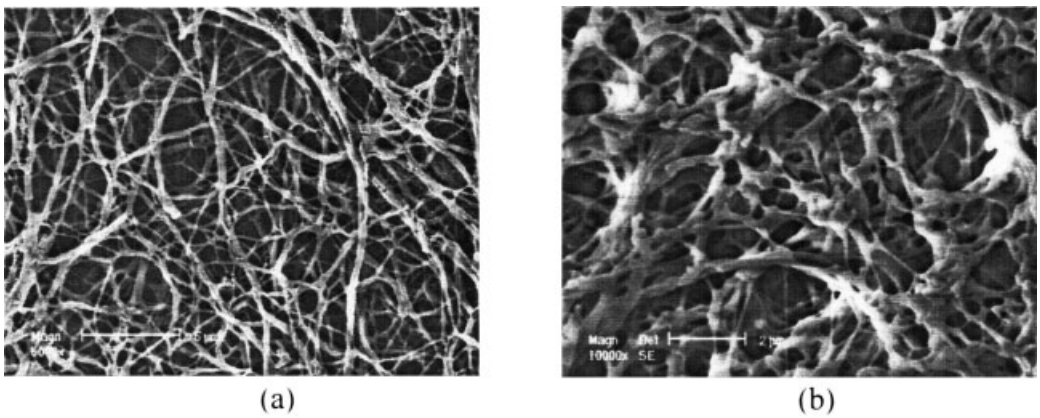


Figure 5 SEM micrographs of scaffolds using distilled water as the exchanging medium at 20°C: (a) PLLA; (b) PLLA/PCL (80/20). (Original magnification ×10,000.)

smoother and more uniform in contrast to the procedure using distilled water; moreover, the microstructure was more regular as well. This suggested that a less polar nonsolvent (e.g., ethanol) was more suitable in this case.

Average diameter and specific surface area of ultrafine fiber within these scaffolds are shown in Table II. The diameter was measured from the SEM micrograph and the specific surface of fiber was calculated with a model described elsewhere.[17] The surface areas of the fiber ends can be neglected because of the very large aspect ratio of the fibers in the continuous fibrous network. Therefore, the specific surface area was obtained from eq. (3):

$$\frac{A_f}{V_f} = \frac{\pi dl}{\pi d^2 l/4} = \frac{4}{d} \tag{3}$$

where d and l are the diameter and length of the fiber, respectively.

It may be seen that the average fiber diameters were about 100–300 nm on the same scale as those of col-

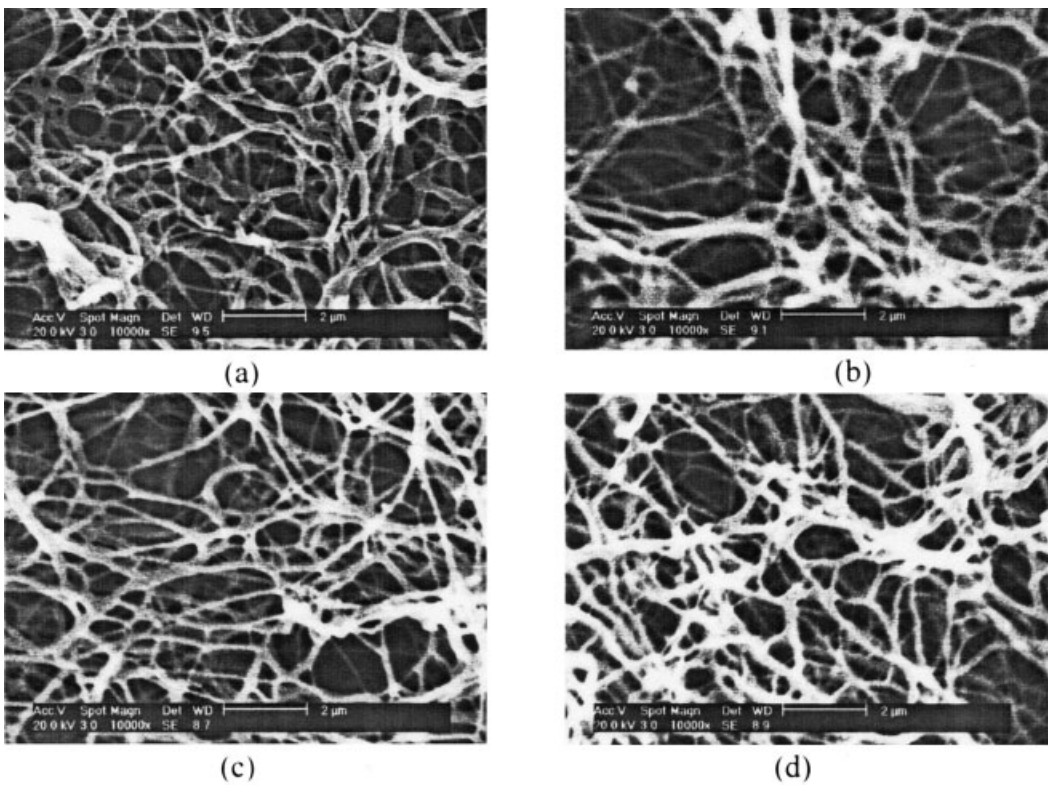


Figure 6 SEM micrographs of PLLA/PCL scaffolds in different mass ratios using ethanol as the exchanging medium: (a) 100/0; (b) 90/10; (c) 80/20; (d) 70/30. (Original magnification ×10,000.)

TABLE II
Average Diameter and Specific Surface Area of Scaffolds Using Water or Enthanol as Exchanging Medium

PLLA/PCL (w/w)	Average diameter/water (nm)	Special surface area/water (m^{-1})	Average diameter/enthanol (nm)	Special surface area/ethanol (m^{-1})
100/0	215 ± 92	1.86×10^7	215 ± 68	1.86×10^7
90/10	214 ± 36	1.87×10^7	196 ± 61	2.04×10^7
80/20	210 ± 73	1.90×10^7	159 ± 29	2.52×10^7
70/30	178 ± 37	2.25×10^7	142 ± 50	2.82×10^7

lagen fibers in an extracellular matrix.[24] The specific surface area was so high that it will potentially facilitate cells to attach. In addition, the diameter and specific surface area of fibers were not influenced either by the change of the exchange medium or by the variation in composition of PLLA and PCL.

Mechanical properties

The compressive moduli of both dry and wet scaffolds are displayed in Figure 7. It was found that with the increase of PCL the compressive moduli of dry samples decreased. Moduli for the polyblend PLLA/PCL compositions of 80/20 and 70/30 were significantly lower than that of the PLLA scaffold ($p < 0.05$). This indicated that the addition of PCL effectively reduced the rigidity of PLLA, which was probably attributable to the rubbery state of PCL at room temperature.[25] Therefore, the flexibility of the fibrous scaffolds was enhanced. That is to say, the incorporation of PCL contributed to a decline in the rigidity of the scaffold and accounted for better flexibility.

After being soaked in PBS for 48 h at 25°C, the compressive moduli of blended scaffolds of different compositions decreased compared to that at dry state (Fig. 7), whereas the degree of reduction was not the same. The difference between these two conditions became increasingly smaller with the decline of m_{PLLA}, which may be attributable to the diffusion of the water molecules into the scaffold surface. Water molecules facilitate the movement of the macromolecule chain segments. Therefore the modulus decreased. Considering that the amounts of the hydroxyl end groups and the ester groups in PLLA are higher than those in PCL, it is more likely for the PLLA to be influenced by water. This caused the difference of the decline among different composition scaffolds.

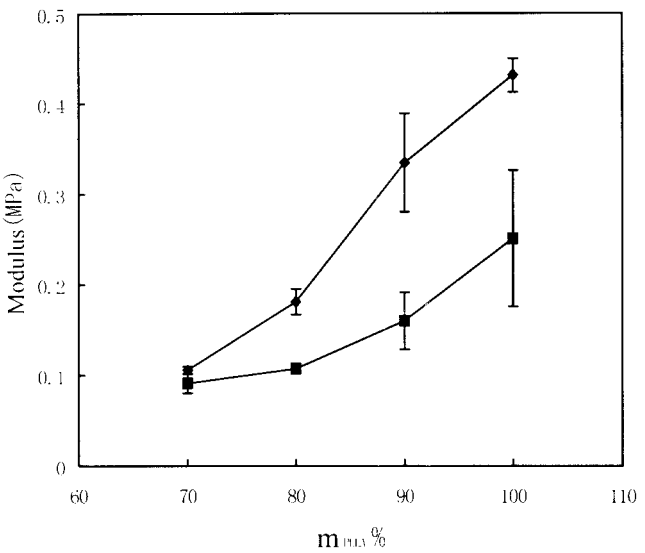


Figure 7 Compressive moduli of scaffolds against PLLA contents (m_{PLLA}) under dry (◆) and wet (■) states.

Cell morphology on the scaffolds

The morphology of chondrocytes cultured on PLLA and PLLA/PCL (80/20) scaffolds was observed under SEM, as shown in Figure 8. After 3 days of culture, chondrocytes adhered to the surface of the scaffolds in significant numbers and separated evenly [Fig. 8(a), (b)]. Cells maintained a spherical phenotype and had a tendency to secrete their own matrix along the direction of the ultrafine fibers. The results indicated that the ultrafine fibrous network structure provided a good environment for cell attachment and was conducive to keeping the cell matrix from leaking. Meanwhile, it may be seen that the degree of matrix secretion by chondrocytes on the PLLA/PCL scaffold was greater than that on the PLLA scaffold [Fig. 8(c), (d)]. This seemed probably to be associated with the mechanical property of the polymer used. PLLA is rigid, whereas PCL is flexible. The force that must be surmounted when the matrix secreted along the fiber might be less on the PLLA/PCL scaffold than that on the PLLA scaffold. Further detailed studies on this phenomenon will be carried out in the future.

CONCLUSIONS

PLLA/PCL blended scaffolds were prepared through the method of gelation, solvent exchanging, and freeze-drying. The scaffolds showed ultrafine fibrous networks with " small block" structure. The diameter and specific surface area of fibers remained un-

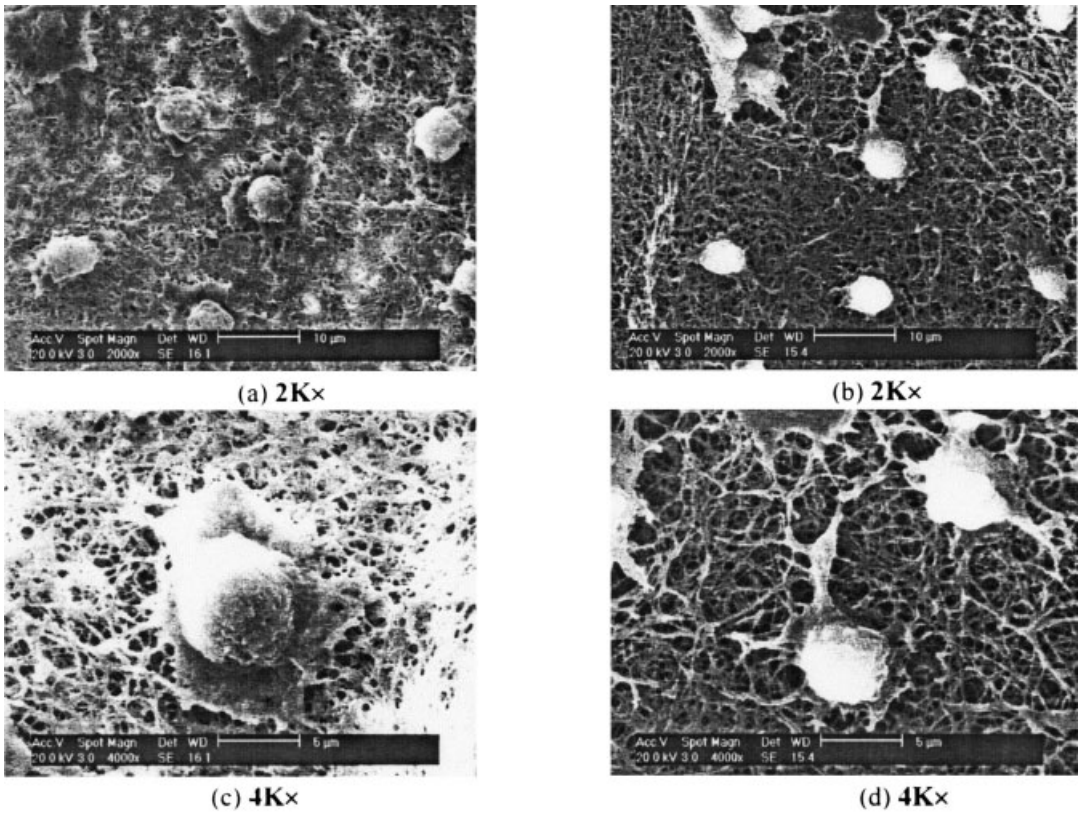


Figure 8 SEM micrographs of cell morphology on PLLA and PLLA/PCL (80/20) scaffolds after 3 days of chondrocytes culture: (a), (c) PLLA/PCL scaffold; (b), (d) PLLA scaffold. [Original magnifications: (a), (b) ×2000; (c), (d) ×4000.]

changed with the addition of PCL. At higher solvent-exchanging temperature (e.g., 20°C), fibrous structure did not exist and the gel network dissociated, caused by the acceleration of the movement of PCL molecular chain segments. The fiber in the scaffolds became smoother and more regular when using ethanol as the exchanging medium. Moreover, PCL effectively reduced the brittleness of PLLA/PCL scaffolds. Chondrocytes cultured on PLLA/PCL scaffold evenly adhered on the fibrous network and presented a satisfactory morphology. The polyblend scaffold would adapt to the potential application in cartilage tissue engineering.

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