Specially Elaborated Thermally Induced Phase Separation to Fabricate Poly(L-lactic acid) Scaffolds with Ultra Large Pores and Good Interconnectivity

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ABSTRACT: Poly(L-lactic acid) (PLLA) scaffolds with pore diameters from several micrometers to ~300 μm were fabricated by a specially elaborated thermally induced phase separation technique. Two different coarsening protocols, i.e., normal coarsening and multi-step coarsening were compared in consideration of phase separation and domain growth. A normal coarsening route produced scaffolds with pore size from several micrometers to 150 μm depending on the coarsening time after phase separation, accompanying with the emergence of isolated pores at long time coarsening. Scaffolds with large pores with size up to ~300 μm were fabricated by the two-step coarsening technique, e.g., the PLLA-solvent (dioxane/water) system was coarsened at a temperature after phase separation for a period, followed by coarsening at a lower temperature for another period. In parallel with formation of the large pores, the interconnectivity between pores was also improved, which was evidenced by scanning electron microscopy, gelatin solution pervasion, and collagen entrapment. The present technique provides the ability to produce scaffolds with high purity, controllable microstructures, and ease of modification, and hence can be widely used in tissue engineering field. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 101: 3336–3342, 2006

Key words: thermally induced phase separation; poly(lactic acid); scaffolds; microstructure

INTRODUCTION

Porous polymer materials have been widely employed as analogues of native extracellular matrix (ECM) in field of tissue engineering such as bone, cartilage, liver, skin, and other tissues.[1–5] To meet the demands of regeneration of different organs, tissue engineering requires the polymer scaffolds with various pore sizes and pore morphology. For example, the optimal pore size for fibroblast ingrowth is about 20 μm, for regeneration of adult mammalian skin is between 20 and 125 μm, while for regeneration of bone is 100–250 μm.[6–8] Therefore, the ability to fabricate porous polymer materials with controllable pore size and porosity is of both methodological and clinical significance.

Several techniques such as porogen leaching, foaming, fiber processing, 3D micro printing, and phase separation have been developed to prepare porous polymer scaffolds. Thermally induced phase separation (TIPS) is one of the practical techniques for fabrication of low-density polymer matrix with controllable properties, which in most cases can meet the demands of tissue engineering and drug delivery.[9–11] The process can be easily controlled and repeated to form different kinds of microstructures, and is applicable to many kinds of soluble polymers.[12] Polymer is dissolved in solvent at elevated temperature. The single homogeneous solution is converted via the removal of thermal energy to phase-separated domains composed of polymer-rich phase and polymer-lean phase. Subsequent freeze-drying of the phase-separated polymer system removes the solvent and produces microcellular structure.[11,13,14]

It has been demonstrated that the coarsening process results in pore size enlargement primarily via Ostwald ripening, coalescence, or hydrodynamic flow mechanism.[13–15] Generally, the coarsening process should be carried out at the temperature that is below the phase separation temperature of the polymer-solution system (upper critical solution system) or polymer crystallization temperature (T_c) but higher than the solidification temperature of the solvent. Figure 1(a)–1(d) presents the normal coarsening process (Route 1). At the beginning of the phase separation,

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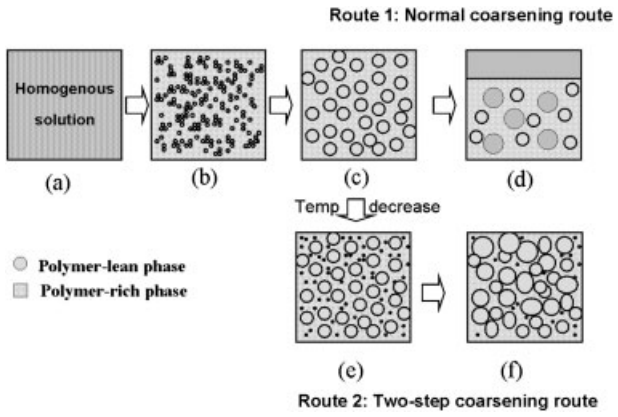

Figure 1 Schematic illustration to show the microstructure change in thermally induced phase separation process. Two coarsening protocols are suggested, i.e., the normal (Route 1) and the multi-step coarsening (Route 2) processes. For detail, see the text.

the polymer-diluent starts to exchange heat with the environment. Lots of polymer-lean droplets appear and coalesce with each other when they become big enough [Fig. 1(b)]. As coalescence process goes on, the droplets turn to be isolated due to the size enlargement and the increase of the system viscosity as a result of polymer concentration increase and crystallization in the polymer-rich domains. This would mean that the droplets must spend more time in crossing through the polymer-rich phase to combine with others. Hence, the growth rate of the droplets is slowed down [Fig. 1(c)]. Before the pores grow large enough, some parts of the polymer-rich phase cannot bear the weight of the polymer-lean droplets, leading to the collapse because of the hydrodynamic flow and gravity. As the result of the sedimentation, a large amount of pores in the isolated state is formed [Fig. 1(d)]. Therefore, it is difficult to fabricate scaffold with pore size larger than 200 μm, while preserving the good interconnection via normal TIPS process. In general, the morphology of the pores turns from open to half-open, and finally to close cellular structure when the pore size is enlarged. The pore size of the polymer scaffolds produced by TIPS so far is below 200 μm.[16] This is a great limitation of the technique that deters its applications on those tissues or organs relying on scaffolds with larger pore size, for example, cartilage and breast.

Recently, some impressing and significative researches have been carried out to solve this problem, such as addition of various additives or electrolytes, which can raise the cloud point temperature of the solution system. Scaffolds with pore diameter larger than 200 μm have been created via a modified TIPS method.[12,17] Different from these existing pathways, herein we introduce a multi-step coarsening route (Route 2 in Fig. 1) in TIPS that may produce scaffolds with larger pore size and good connectivity, while avoiding the introduction of other components. The basic idea is that after phase separation the polymer–solvent system is coarsened at a higher temperature for a period of time. The system is then further decreased to a lower temperature and coarsened again for another period before freeze-drying. By such a protocol, the first coarsening has converted the system to polymer-rich phase and polymer-lean phase [Fig. 1(c)]. After decreasing the system to a lower temperature, some new polymer-lean domains can be formed in the polymer-rich phase [Fig. 1(e)] because of the supersaturation of these regions. These newly formed and the initially existed polymer-lean domains may grow, fuse, coalesce, and accumulate with each other to form larger domains [Fig. 1(f)], which correspond to the cavities after solvent removal. By controlling the time of the phase separation in the secondary coarsening, this method may provide the possibility to simultaneously prepare polymer scaffold with large pore size and good interconnectivity.

To demonstrate this idea, a typical biodegradable polymer, poly(L-lactic acid) (PLLA), is selected, employing dioxane/water as a solvent system. Long time coarsening at higher temperature is first applied for the sake of pore enlargement, followed by a second coarsening at lower temperature. With this two-step coarsening protocol, PLLA scaffolds with open cellular structure are obtained. Their pore size can be readily controlled from several micrometers to ~300 μm. It will be shown that this innovation can be greatly helpful in scaffold fabrication with well controlled microstructure.

METHODS

Materials

The PLLA (M_n = 200,000, M_w = 400,000) was synthesized using the method described previously.[18] 1,4-Dioxane was a product of Shanghai Chemical Industries Co. Ltd, China, and used as received.

Cloud point determination

A mixture of 1,4-dioxane/triple-distilled water (87/13, v/v)[11,17] was used to dissolve PLLA at 80°C. A microscope was used to follow the appearance of the turbidity of the PLLA solutions with various concentrations (w/v) as a result of temperature decrease at a rate of 10°C/min. Cloud points were determined visually by the emergence of turbidity under the microscope.

Scaffold preparation

Route 1. The PLLA solution in a glass mold (1.2 cm in diameter and 10 mm in thickness) at 70°C was quickly

quenched to 37 or 25°C and incubated for 0–8 h to induce coarsening effect.[14] The system was then quenched at −20°C for 1 h before solvent removal by freeze-drying.

Route 2. After coarsening at 25°C for 5 h as mentioned before, the phase separated system was cooled down to 20°C and coarsened again for a given time before freeze-drying.

Pore size and interconnectivity determination

The PLLA porous scaffolds were fractured in liquid nitrogen. Their morphology in the cross sections is observed under a scanning electron microscope (JEOL JEM). The diameter of the pores was determined and averaged from the SEM images by graph calculation software, SMile view.

Porosity

Since the macroscopic shape of the scaffolds is regular and their volume (V) can be easily measured, the porosity of the scaffolds was calculated by the following equation:

$$\text{Porosity} = (V - W/\rho)/V \times 100\%$$

where W is the weight of the scaffolds and ρ is the density of PLLA, 1.27 g cm^{-3} measured by weight–volume method.[19]

Differential scanning calorimetry (DSC)

The thermal properties of the PLLA scaffolds, including melting points and corresponding enthalpy changes, were measured by a Perkin–Elmer DSC 7 calorimeter. The samples (between 2 and 4 mg) were heated at a rate of 10°C/min. The peak temperature of melting endotherm was recorded as T_m. The intrinsic degree of crystallinity (X_c) was calculated from the following equation:

$$X_c = \Delta H_m / \Delta H_m{}^0$$

where ΔH_m is the melting enthalpy of the measured PLLA and $\Delta H_m{}^0$ is the melting enthalpy of 100% crystalline PLLA (203.4 J/g).[20]

Gelatin loading

The scaffolds prepared via Route 1 (8 h coarsening at 25°C) and Route 2 (3 h secondary coarsening) were damped first by 75% alcohol solution for 1 day, followed by exchanging the alcohol with phosphate buffered saline (PBS, pH 7.4) for 12 h. The prewetted scaffolds were incubated into 1% gelatin solution for 2–90 min. The loaded gelatin was quantified as follows after freeze-drying. The scaffolds were completely hydrolyzed with 6*M* HCl for 12h at 120°C. The content of the gelatin in the scaffolds was determined by measuring the hydroxyproline content spectrophotometrically referring to the method of Cheung et al.[21]

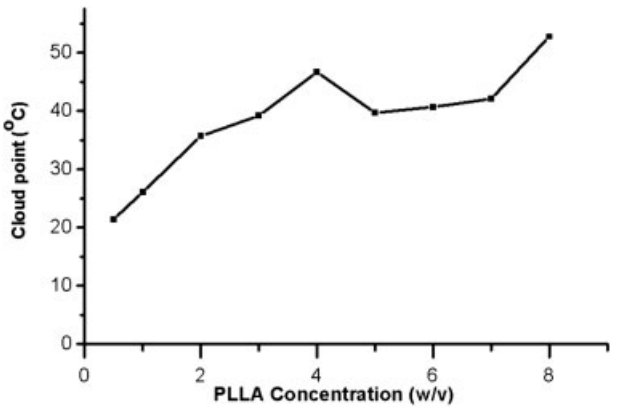

Figure 2 Cloud point curve for PLLA in 1,4-dioxane/water (87 : 13 v/v) solution system. Weight–volume concentration was used.

Collagen introduction

Type I collagen solution (1.5–2.5 mg/mL, in 0.3 vol % acetic acid) was pressed into the scaffolds by a negative pressure method.[22–26] Briefly, after evolved the trapped air bubbles from the internal scaffolds under reduced pressure, the pressure was released to the ambient value. The collagen solution was spontaneously pressed into and entrapped inside the scaffolds. After repeating for 3 times, the collagen-loaded scaffolds were freeze-dried. Quantification of the collagen amount was similarly conducted with that of gelatin.

RESULTS AND DISCUSSION

Phase diagram

By varying the preparation condition, many kinds of desired scaffold structure can be manufactured via TIPS method. Therefore, it is important to know the phase diagram first, so that phase separation can be induced by reducing the casting temperature to a temperature lower than the upper critical solution temperature (UCST) or crystallization temperature. However, a precise plotting of the phase diagram is difficult because of the polydispersity of the macromolecules. Hence, a cloud point curve, which is very close to the real phase diagram, is used to depict the critical phase separation temperature (T_c) and the critical polymer concentration.[24] The cloud-point temperatures of the PLLA solutions are presented in Figure 2. Similar with the previous results,[11,17,18] the cloud-point temperature elevated with the increase of poly-

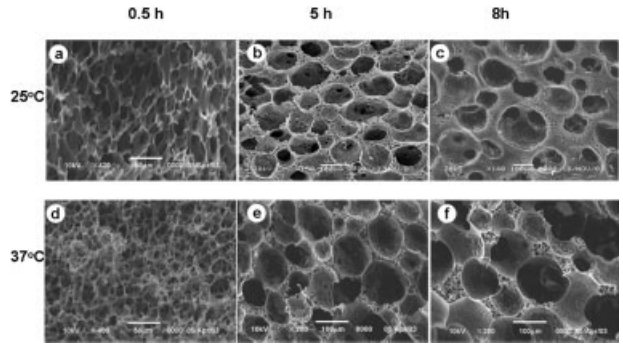

Figure 3 SEM images to show the cross sections of the PLLA scaffolds as a function of coarsening temperature and coarsening time. Coarsened for (a) and (d) 0.5 h, (b) and (e) 5 h, and (c) and (f) 8 h, and at (a–c) 25°C and (d–f) 37°C.

mer concentration. The small peak at about 4% shows that the composite is a UCST system. Previous research also indicates that sedimentation is incidental when the PLLA concentration is below the boundary concentration (about 4.5 wt %). This will lead to the formation of two layers during the quenching process.[11,17,19,27] Hence, a slight concentrated PLLA solution, e.g., 5%, was used to fabricate the macroporous PLLA scaffolds due to the mechanical consideration and the ability to resist collapse in practical applications.

Coarsening time and temperature

As shown in Figure 3, regular round pores were generated in the sponge with highly interconnected microstructure in particular at short coarsening time, proving that the phase separation follows a spinodal mechanism via Route 1. The porosity of the scaffolds is about 93%, which is slightly smaller than that of theoretically calculated (i.e., 95%) because of the partial collapse. Statistical results revealed that the diameters of the pores increased as the coarsening time was extended (Fig. 4). The growth rate of the pore size was very fast from 0 to 2 h because of formation of the polymer-lean droplets and the rapid coalescence. After 3 h, the pore size grew to ~100 μm and the droplets became somewhat isolated. Hence, the pore growth rate was decreased. A slower growth rate of the pore size was found from the coarsening time of 3–5 h. After that, the growth rate became faster again accompanying with formation of more isolated pores. Along with the continuous extending of the polymer-lean phase, the structure of some parts of the polymer-rich phase is not tough enough to bear the weight of the growing droplets, and thus collapsed. Observations under SEM (Fig. 3) confirm that the morphology of the pores turns from open cellular structure to half-open cellular structure when the coarsening time prolongs. Moreover, Figures 3 and 4 prove that the coarsening temperature has minimal influence on the growth of the pore size compared with coarsening time. The largest pore size was only ~150 μm even after coarsened for 8 h by Route 1. At this moment half-open pore morphology emerged too [Fig. 3(f)].

We have to mention that the present coarsening is different from the traditional fast quenching procedure. In the latter case, quenching temperature is usually regarded as a crucial factor affecting significantly the microstructure, since the pore size is determined by the growth rate of polymer-lean phase and the time of phase separation. Usually, the system is rapidly decreased below the frozen temperature (T_f), at which phase separation stops. Therefore, a faster quenching rate will generally produce smaller pore size. However, in the present case, no significant difference was found between the samples quenched from 25 or 37°C to −20°C in all the time intervals. The reason should be that the long enough coarsening at a temperature above the T_f has created very large extent of phase separation. Consequently, the contribution of the latter fast quenching is comparatively small and neglectable for the present protocol, as also evidenced later.

Effect of two-step coarsening

The ability to fabricate scaffolds with various microstructures such as pore size and porosity is crucial for construction of tissues relying on the supports. Through the normal TIPS process, i.e., Route 1, scaffolds with pore size from several micrometers to 150 μm can be produced as illustrated earlier. Moreover, lots of tiny pores are observed in the walls of the large pores after coarsened for longer time [Figs. 3(c) and 3(f)]. This phenomenon is attributed to the secondary phase separation during fast quenching (or freezing).[11,17,18,28] However, because the time of the sec-

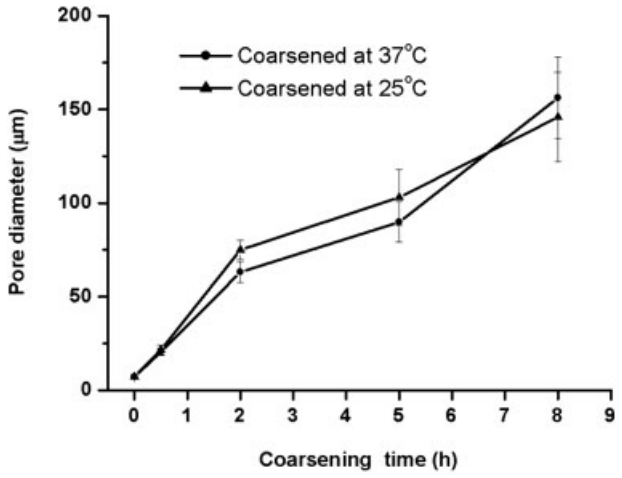

Figure 4 The average pore diameter as a function of coarsening time at 37 or 25°C.

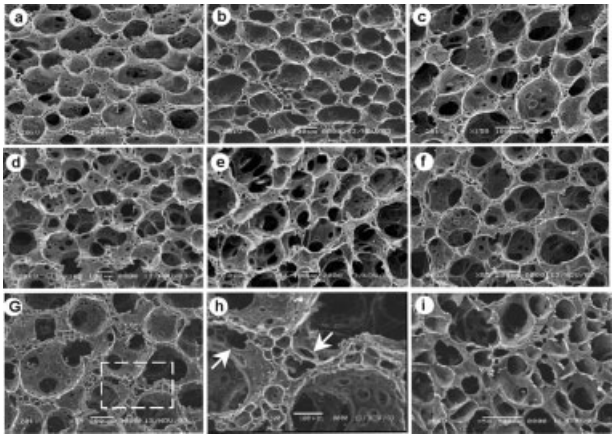

Figure 5 SEM images to show the cross sections of the scaffolds fabricated by the two-step coarsening (Route 2). The PLLA-1,6-dioxan/water (87/13) system was first coarsened at 25°C for 5 h, and then coarsened at 20°C for (a) 0, (b) 15, (c) 70, (d) 90, (e) 110, (f) 150, (g) 180, and (i) 240 min. (h) is higher magnification of (g).

ondary phase separation is too short, the resultant tiny pores contribute little to enlargement of the pore size. To fabricate scaffolds with still larger pore size by TIPS, herein a multi-step coarsening process (Route 2 in Fig. 1) is suggested. By this process, no other additives are required. As will be shown later, the good interconnectivity of the pores is also preserved accompanying with enlargement of the pore size.

The basic process is demonstrated by successively coarsening the polymer–solvent system at two temperatures (two-step coarsening) after phase separation. The system was first coarsened at 25°C for 5 h to form the polymer-rich and the polymer-lean domains as described earlier. The phase-separated system was then cooled down to 20°C and coarsened again for up to 4 h before freeze-drying. By such a protocol, some new polymer-lean domains could be formed in the initial polymer-rich domains because of the supersaturation of these regions. These newly formed and the initially existed polymer-lean domains may grow, fuse, coalesce, and accumulate with each other to form larger domains, which correspond to the cavities after solvent removal. The SEM observations confirm the efficacy of this idea (Fig. 5). In the first 60 min of the second coarsening, the rate of pore enlargement did not increase obviously, because the newly formed droplets were not big enough to increase the coalescence of the original polymer-lean droplets. As the time went on, the rate of pore enlargement increased obviously because the size of the newly formed droplets became large enough and the polymer-rich phase became easy to be crossed over. The average pore diameter had reached about 320 μm after 3 h and some pores with a diameter above 400 μm had appeared [Fig. 5(g)]. As a comparison, the pore size of the scaffold produced with the same coarsening time (total 8 h) at 25°C is only 140 μm (Fig. 4).

Besides the enlargement of the pore size, there are other differences brought by Route 2 as well. For example, the interconnectivity of pores created by Route 2 is considerably improved in comparison with that by Route 1, as can be drawn by comparison of Figures 5(g) and 3(c). Figure 5(h) shows the details of pore wall structure. The pore walls have many small pores as the scaffold prepared via Route 1, but the amount of these little pores was comparatively decreased and the pore size became much larger (10–50 μm). This phenomenon provides an evidence for the accumulation and coalesces of the newly formed polymer-lean domains. Many interconnecting regions [indicated by the arrows in Fig. 5(h)] appeared in the pore walls when the second coarsening time was long enough [Fig. 5(c–i)], which conveys a hint that the newly formed smaller pores have bridged between the initially formed larger pores. These results have actually provided an indirect proof that the supposed mechanism for the two-step coarsening (Fig. 1, Route 2) is basically correct. Different from the addition of additives or electrolytes, this two-step coarsening only tunes the evolution of phase separation instead of improving the cloud point of the system.

PLLA is a semicrystalline polymer. It has been reported that crystallization behavior can be used to tune the pore size of the scaffold in a certain range.[11,17,28] In this research, however, the crystallization behavior of the PLLA was not deliberately controlled. The DSC thermograms show that PLLA in all the scaffolds is in a semicrystalline state, with a melting point within 170–171°C and a crystallization degree around 11–13% (Fig. 6 and Table I). Detail anal-

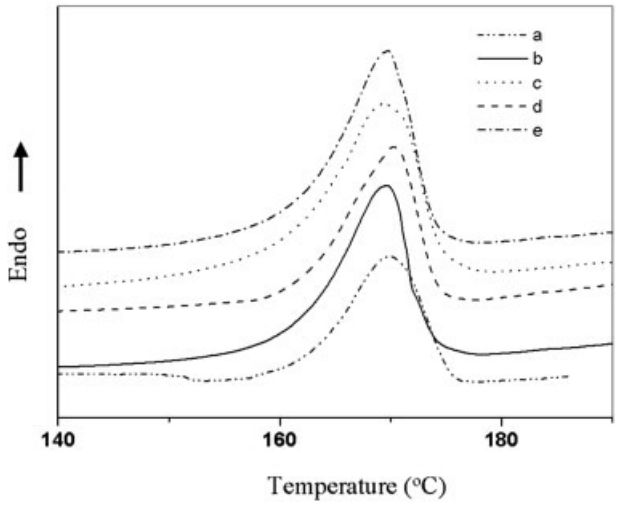

Figure 6 DSC thermograms of the PLLA scaffolds prepared via Route 1 and Route 2 at different conditions. The results are summarized in Table I, wherein sample designation can be found too.

TABLE I
Melting Temperature and Crystallization Degree of PLLA Scaffolds Measured by DSC

Scaffold	Primary coarsening time (h)	Secondary coarsening time (h)	T_m °C	ΔH_m (J/g)	X_c (%)
a	1	–	170.1	22.33	10.9
b	5	–	169.7	23.17	11.3
c	8	–	169.5	26.43	12.9
d	5	1	170.5	23.35	11.4
e	5	3	169.9	26.77	13.1

ysis reveals that the crystallization degree of the PLLA scaffolds (Table I) increased with extension of coarsening time, implying that the PLLA molecules in gel-like polymer-rich domains were still movable before the solvent solidification. This might also be a reason that the phase separation can only reach some extent by Route 1.[27–29] On the other hand, the same crystallization degree at a total coarsening time of 8 h, regardless of coarsening route, implies that the crystallization should not be a crucial factor affecting the pore size in Route 2, but the combination and coalescence of the newly formed polymer-lean domains are.

Protein loading

The loading capacity of the PLLA scaffolds was further checked by using gelatin (MW 40,000) as a model substance. This is, on the one hand, important to incorporate bioactive proteins or cell growth factors, and on the other hand, may provide further evidence to compare the interconnectivity of the scaffolds. For this purpose, the scaffolds fabricated by Route 1 and Route 2 at the same coarsening time (total 8 h) were selected. Figure 7 shows that the amount of gelatin incorporated in the scaffold prepared by Route 2 was always higher in all time points and eventually reached 2 times that of the scaffold prepared by Route 1. Since the scaffolds possess roughly same apparent density ρ_a (0.0952 and 0.09 1 g/cm^3 for scaffolds fabricated by Route 1 and Route 2, respectively), this result would mean that the scaffold fabricated by Route 2 should have better interconnectivity between pores. Taking into account the porosity of the scaffold (porosity = $1 - \rho_a/\rho_{PLLA}\rho_{PLLA} = \sim 1.27$ g/cm^3, the density of PLLA[19]) and the gelatin concentration (1% = 10 mg/mL), the maximum amount of gelatin that can be theoretically introduced is calculated as 9.3 mg/cm^3 scaffold. Hence, the introduced gelatin amount even for the scaffold fabricated by Route 2 is still much lower than the theoretical value, which should be attributed to the existence of closed pores or high viscosity of the biomacromolecule solution.

The better interconnectivity was further confirmed by introduction of more sticky collagen solution into the porous scaffolds by a method of negative pressing.[22] Again, the scaffold prepared by Route 2 exhibited higher loading capacity at the same collagen concentration (Table II). The >100% loading capacity is caused by adhesion of the collagen molecules on the scaffold surfaces. It is well-known that both gelatin and collagen are biocompatible biomacromolecules that can enhance cell–material interaction, thus loading of these substances would be of course beneficial to modify the resultant scaffolds.

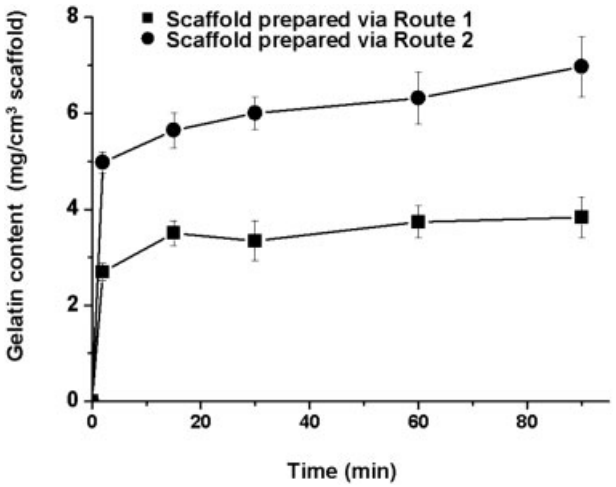

Figure 7 The introduced gelatin amount as a function of incubation time. PLLA scaffold fabricated by only coarsened at 25°C for 8 h (Route 1 scaffold) or by first coarsened at 25°C for 5 h and then coarsened at 20°C for 3 h (Route 2 scaffold).

CONCLUSIONS

We have shown here that PLLA scaffolds with average pore diameters from several micrometers to >300μm can be produced by specially elaborated thermally induced phase separation (TIPS) technique without involvement of any additives or electrolytes. Two different coarsening techniques are elucidated in consideration of the phase separation and domain growth. A normal coarsening process (Route 1) can yield PLLA scaffolds with an average diameter from several micrometers to ~150 μm depending on the coarsening time, accompanying with the emergence of

TABLE II
Collagen Content in the PLLA Scaffolds

Collagen concentration (mg/mL)	Preparation process	Collagen content (mg/cm^3 scaffold)
1.5	Route 1	1.4 ± 0.2
2.5	Route 1	3.0 ± 0.3
1.5	Route 2	2.2 ± 0.3
2.5	Route 2	3.8 ± 0.4

closed pore morphology. A multi-step coarsening technique (Route 2) is described, showing that it is powerful to produce scaffolds with ultra large pore size and good interconnectivity between pores. The two-step coarsening and quenching endows the feasibility to tailor and modulate the pore size of the resultant scaffolds. Together with other intrinsic features of the technology, the present method makes the technique be more versatile and suitable for fabricating scaffolds that may be used in various tissue-engineered organs. Exemplified by gelatin introduction and collagen loading, the good interconnectivity of the macroporous PLLA scaffolds has been illustrated.

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