Porous biodegradable polymeric scaffolds prepared by thermally induced phase separation

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Abstract: Poly(L-lactic acid) and its copolymers with D-lactic and glycolic acid were used to fabricate various porous biodegradable scaffolds suitable for tissue engineering and drug delivery based on a thermally induced phase separation (TIPS) technique. A variety of parameters involved in TIPS process, such as types of polymers, polymer concentration, solvent/nonsolvent ratio, and quenching temperature, were examined in detail to produce a wide array of micro- and macroporous structures. A mixture of dioxane and water was used for a binary composition of solvent and nonsolvent, respectively. In particular, the coarsening effect of pore enlargement affected by controlling the quenching temperature was used for the generation of a macroporous open cellular structure with pore diameters above 100 μm. The use of amorphous polymers with a slow cooling rate resulted in a macroporous open cellular structure, whereas that of semicrystalline polymers with a fast cooling rate generated a microporous closed cellular structure. The fabricated porous devices loaded with recombinant human growth hormone (rhGH) were tested for the controlled delivery of rhGH, as a potential additional means to cell delivery. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res, 47, 8–17, 1999.

Key words: biodegradable polymer; porous scaffolds; thermally induced phase separation

INTRODUCTION

Poly(lactic acid) (PLA) and poly(D,L-lactic-*co*-glycolic acid) (PLGA) have been widely used as three-dimensional (3D) polymer scaffolds for cell transplantation, since they are biodegradable and biocompatible.[1] It is important that the scaffolds be highly porous enough to allow a high density of cells to be seeded, and when the cell-device construct is implanted in the body, they should permit the facile invasion of blood vessels for the supply of nutrients to the transplanted cells.[2] To fulfill these requirements, the polymer scaffolds should have several physical characteristics such as high porosity, a large surface area, a large pore size, and a uniformly distributed and highly interconnected pore structure throughout the matrix.[3,4] Until now, nonwoven polyglycolic acid (PGA) fiber mesh reinforced by physical bonding of PLA and PLGA between the fibers has been the most popularly used scaffold for soft-tissue cell transplantation because of its highly porous and interconnected structure.[3,5,6] However, the PGA nonwoven mesh is not suitable for the purpose of hard-tissue regeneration such as in bone, since it is mechanically fragile.

Several methods have been reported to fabricate porous scaffolds by using biodegradable PLA or PLGA. They are porogen leaching,[5–7] emulsion freeze-drying,[10] expansion in high pressure gas,[11,12] 3D printing,[13] and phase separation techniques.[14–16] The porogen-leaching method has been the best known method, and involves the casting of a polymer/porogen composite membrane followed by aqueous washing out of the incorporated porogen. Various porogens such as salts, carbohydrates, and polymers can be used to produce porous materials. The salt-leaching technique was suitable for controlling pore sizes by changing the size of salt particulates, but residual salts remaining in the scaffolds, irregularly shaped pores, and their poor interconnected structure seem to be problematic for cell seeding and culture. The emulsion freeze-drying method is another approach for the production of porous scaffolds, but this method often results in a closed cellular structure in the matrix. The expansion technique using a high pressured CO_2 gas also resulted in a closed pore structure inadequate for efficient cell seeding, and a combination technique of gas-induced foaming and particulate leaching was re-

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cently proposed to improve the pore structure.[12] The 3D printing technique using the principle of ink-jet printing appears useful, while its application for tissue engineering is still in infancy.[13]

The phase separation technique is based on thermodynamic demixing of a homogeneous polymer-solvent solution into a polymer-rich phase and a polymer-poor phase, usually by either exposure of the solution to another immiscible solvent or cooling the solution below a binodal solubility curve. Thermally induced phase separation (TIPS) uses thermal energy as a latent solvent to induce phase separation.[17,18,27] The quenched polymer solution below the freezing point of the solvent is subsequently freeze-dried to produce porous structure. One of the advantages of this technique is that various porous structures can be easily obtained by adjusting various thermodynamic and kinetic parameters. The TIPS technique has been used commercially to produce microporous membranes for filtration, but the pore size of the resultant membrane is too small to be applied for cell seeding, which requires a pore diameter at least above 100 μm with an open cellular morphology. The TIPS technique has been previously reported for the preparation of porous PLA scaffolds intended to use as tissue scaffolds.[16] Although a wide array of microporous (1–10 μm) isotropic morphologies were reported in response to a slight change in the TIPS parameters, whether they have macroporous and open cellular structure throughout the matrix was not discussed. Large pore size and an open cellular structure are critical parameters for cell seeding and neovascularization when implanted *in vivo.*

In this study, we investigated TIPS technique to fabricate 3D porous biodegradable polymer scaffolds with the aim of attaining macroporous structure with an open cellular morphology. To achieve this goal, we carefully studied the late stage of the phase separation.[19–22,26] After the early-stage development of the microstructure in a phase-separating polymer solution, the system continues to change in response to its tendency to reduce the interfacial free energy between the two phases already separated in the earlier stage. This process, called a coarsening effect, results in an increase in the size of phase-separated droplets with a concomitant reduction in their numbers. The main focus of this study was to use the coarsening process of the phase separation for the preparation of macroporous scaffolds still having an open cellular structure ideal for cell seeding. For this purpose, several experimental parameters such as polymer type and concentration, solvent/nonsolvent ratio, and quenching temperature were varied to optimize the morphology of biodegradable polymer scaffolds suitable for tissue regeneration. In addition, recombinant human growth hormone (rhGH) was loaded into the scaffolds by an immersion-soaking method to evaluate the pore interconnectivity, as well as to demonstrate the possible capability of controlled drug delivery.

MATERIALS AND METHODS

Materials

Poly(L-lactic acid) (PLLA), poly(D,L-lactic acid) (PDLLA), and poly(D,L-lactic-*co*-glycolic acid, lactide:glycolide ratio 85:15 (PLGA) were purchased from Medisorb (Cincinnati, OH). Weight average molecular weights and polydispersity (PI) were determined by gel-permeation chromatography; M_w = 59,000 and PI = 2.07 for PLLA, M_w = 74,500 and PI = 2.07 for PDLLA, and M_w = 74,700 and PI = 1.62 for PLGA. A phenogel 00H-0445-K0 column (300 × 7.8 mm) was used as a size exclusion column. Tetrahydrofuran (THF) was eluted as a mobile phase and the sample was detected by using an ultraviolet (UV) detector at 220 nm. rhGH was obtained as a gift from Dong-A Pharm. Co., Ltd. (South Korea). The microbicinchoninic acid (BCA) assay kit was obtained from Pierce. 1,4-Dioxane, methanol, acetic acid, and other reagents were of analytical quality.

Cloud point and sol/gel transition curve determination

The polymer solution was prepared at various concentrations in an 87/13 (v/v) mixture of 1,4-dioxane and water, and warmed up to at least 10°C above the expected cloud point. The cloud point and sol/gel transition temperatures were then determined visually by slowly cooling the polymer solution by 1°C. The temperature was maintained constant for 1 h for each visual observation.

Scaffolds preparation

Processing condition 1

To observe the structure resulting from the nucleation and growth process of the polymer-rich phase, 1% (w/v) polymer concentration in a mixture of dioxane and deionized water was chosen. Water was added as a nonsolvent to encourage phase separation. The volume ratio of dioxane and water was set at 87:13. The clear polymer solution dissolved above the cloud point was poured into an aluminum foil mold (1 × 1 cm) which was fast-frozen in liquid nitrogen tank or freezer (−15°C) and then incubated at those temperatures overnight. Solvent was removed by freeze-drying for 3 days.

Processing condition 2

To compare the structures resulting from the spinodal decomposition and coarsening process, 500 mg of PLLA, PDLLA, or PLGA polymer dissolved in 5 mL of a mixture of dioxane and water was warmed up above the cloud point until the clear solution was formed. The volume ratios of dioxane and water were varied at 84:16, 87:13, and 90:10. The homogeneously clear solutions were quenched under the

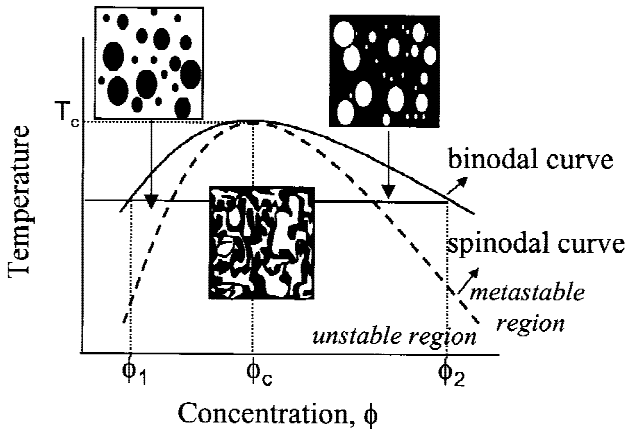

Figure 1. Schematic temperature-composition phase diagram of polymer solution.

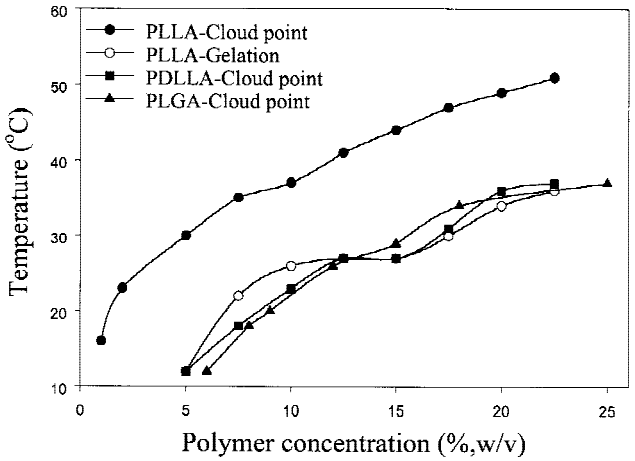

Figure 2. Cloud point curves and sol/gel transition curve of the PLLA, PDLLA, and PLGA polymer solutions in a mixture of dioxane and water with an 87/13 volume ratio.

following conditions: liquid nitrogen bath, deep-freezer (–40°C), and freezer (–15°C), and then incubated overnight at the quenching temperature. Solvent was removed by freeze-drying for 3 days.

Scanning electron microscopy (SEM) and mercury intrusion porosimetry

The internal pore structures of the freeze-dried scaffolds were observed using SEM (Philips 535M). The samples were fractured by a surgical blade and mounted on an aluminum stub covered with a carbon adhesive and then coated with gold particles. Pore size distribution, total pore volume, surface area, and density of the scaffolds were determined by mercury intrusion porosimetry (Porous Materials, Ithaca, NY). The technique is based on the principle that the external pressure required to force a nonwetting liquid (mercury) into a pore, against the opposing force of the liquid surface tension, depends on the pore size (the following Washburn equation)[23]:

$$P = -\frac{4\sigma \cos \theta}{d}$$

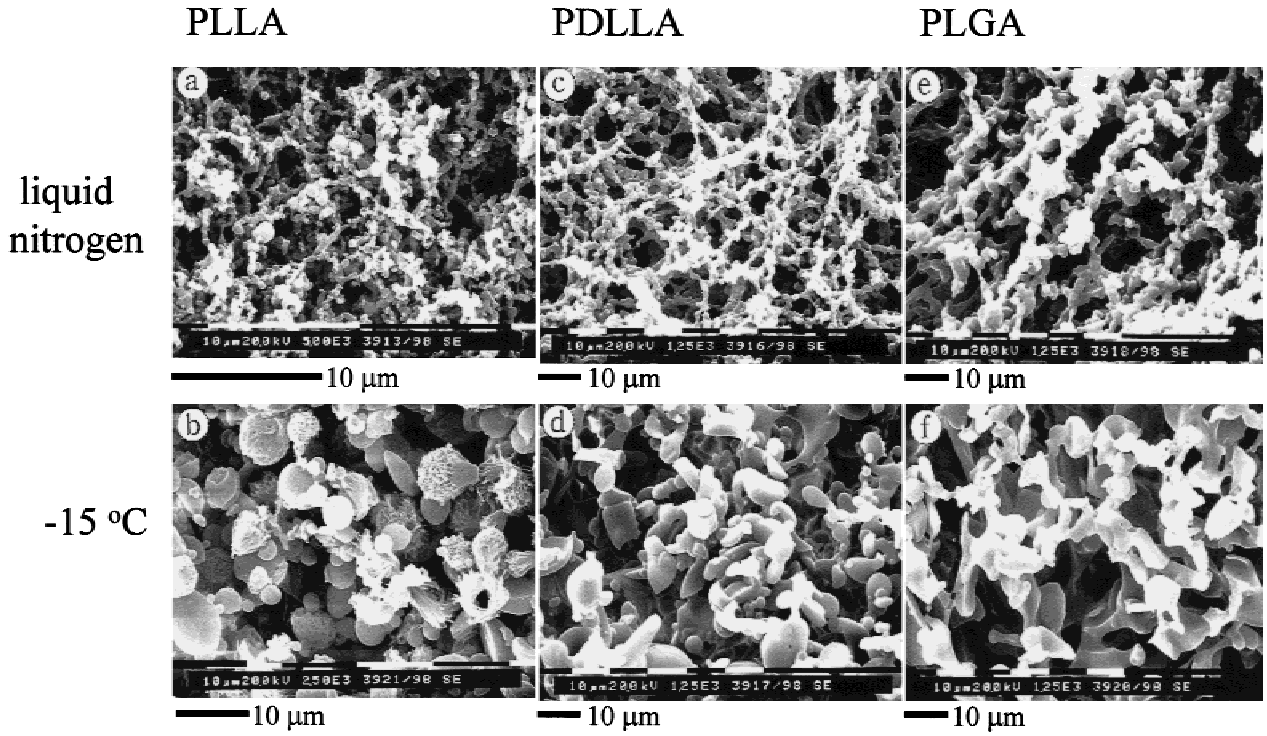

Figure 3. SEM images of foam structures fabricated by using 1% (w/v) PLLA, PDLLA, and PLGA polymer solutions in a mixture of 87/13 (v/v) dioxane/water. Polymer types: PLLA (a,b); PDLLA (c,d); PLGA (e,f). Quenching conditions: liquid nitrogen (a,c,e); –15°C (b,d,f).

which describes the relationship between the applied pressure, P, and the pore size, to calculate the pore diameter, d, under the following assumptions: (a) the shape of the pores is a cylinder; (b) the contact angle of mercury onto pore wall, θ, is 140°; and (c) the surface tension of mercury, σ, is 480 dyne/cm.

Protein loading, distribution analysis, and release experiment

Polymer scaffolds were cut into a dimension 5 × 5 × 4 mm. They were prewetted in pure ethanol and were stirred at 100 rpm for 2 h using an orbital shaker; ethanol was replaced by excess water with continuous shaking.[24] rhGH was then loaded into the prewetted scaffolds by dipping the scaffolds into the protein solution with a concentration of 4 mg/mL at room temperature for 38 h. Loading quantities were determined by measuring the residual amount of protein in the solution. Protein-loaded scaffolds were then rinsed by distilled water and put into the Coomassie blue solution, which consisted of 0.1% (w/v) Coomassie blue R-250, 45% (v/v) methanol, 45% (v/v) water, and 10% (v/v) glacial acetic acid. After incubation for several hours, Coomassie blue destaining was accomplished by dipping the scaffolds into the destaining solution consisting of 10% (v/v) methanol, 10% (v/v) glacial acetic acid, and 80% (v/v) water. The *in vitro* release experiment was accomplished in 33 m*M* phosphate-buffered saline (PBS, pH 7.4, 0.1*M* NaCl) under static conditions at 37°C. The incubation medium was replaced by fresh buffer at predetermined time intervals to maintain a constant pH value. The released protein amount was determined by the micro-BCA protein assay method.

RESULTS AND DISCUSSION

Figure 1 shows a schematic temperature-composition phase diagram for a binary polymer/solvent

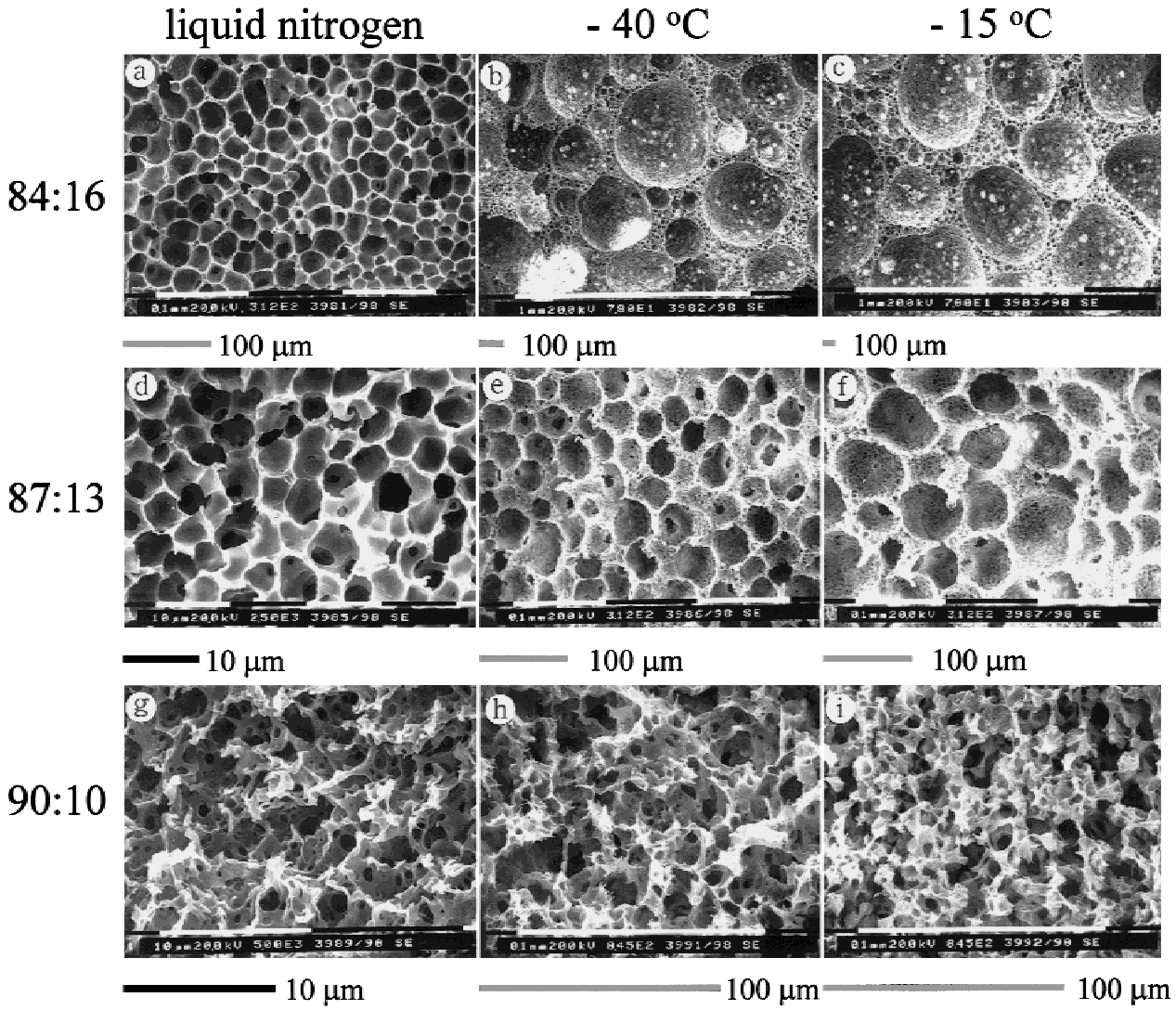

Figure 4. SEM images of the cross section of PLLA scaffolds as a function of coarsening time and solvent/nonsolvent volume ratio. The scaffolds were prepared by quenching a 10% (w/v) polymer solution under the following conditions: liquid nitrogen (a,d,g); –40°C (b,e,h); –15°C (c,f,i). The volume ratios of dioxane and water were 84/16 (a–c); 87/13 (d–f); 90/10 (g–i).

system. Above the binodal curve, a single polymer solution phase is formed, and if cooling below the curve, polymer-rich and polymer-poor phases are separated in a thermodynamic equilibrium state. The spinodal curve is defined as the line at which the second-derivative Gibbs free energy of mixing is equal to zero, and it divides the two-phase region into an unstable and a metastable region. If the system is quenched into the metastable region, phase separation occurs in a nucleation and growth mechanism, leading to a beadlike or isolated cellular structure. On the other hand, if the system temperature is quenched into the unstable region, the phase separation takes place in a spinodal decomposition mechanism, resulting in a microporous interconnected structure as reported.[19,20] These events occur in the early stage of phase separation, but in the later stage, the coalescence of phase-separated droplets continuously proceeds to minimize the interfacial free energy associated with the interfacial area, which is called the coarsening process. This effect has been scrutinized in the fabrication of synthetic membranes, because it is vitally important in determining the final membrane morphology.[21,22] To attain macroporous scaffolds, it is highly desirable to use the coarsening effect, which induces the enlargement of pores. The coarsening process, however, concomitantly tends to generate more closed pores; thus, it is important to optimize various TIPS parameters to achieve a macroporous open cellular structure.

Various biodegradable polymers, PLLA, PDLLA, and PLGA (85/15), dissolved in a solvent/nonsolvent mixture of dioxane/water were used for the generation of liquid–liquid phase separation as reported.[25] The reason for selecting dioxane as a solvent for biodegradable polymers is that it has a low boiling point, 101.1°C, with a relatively high melting temperature,

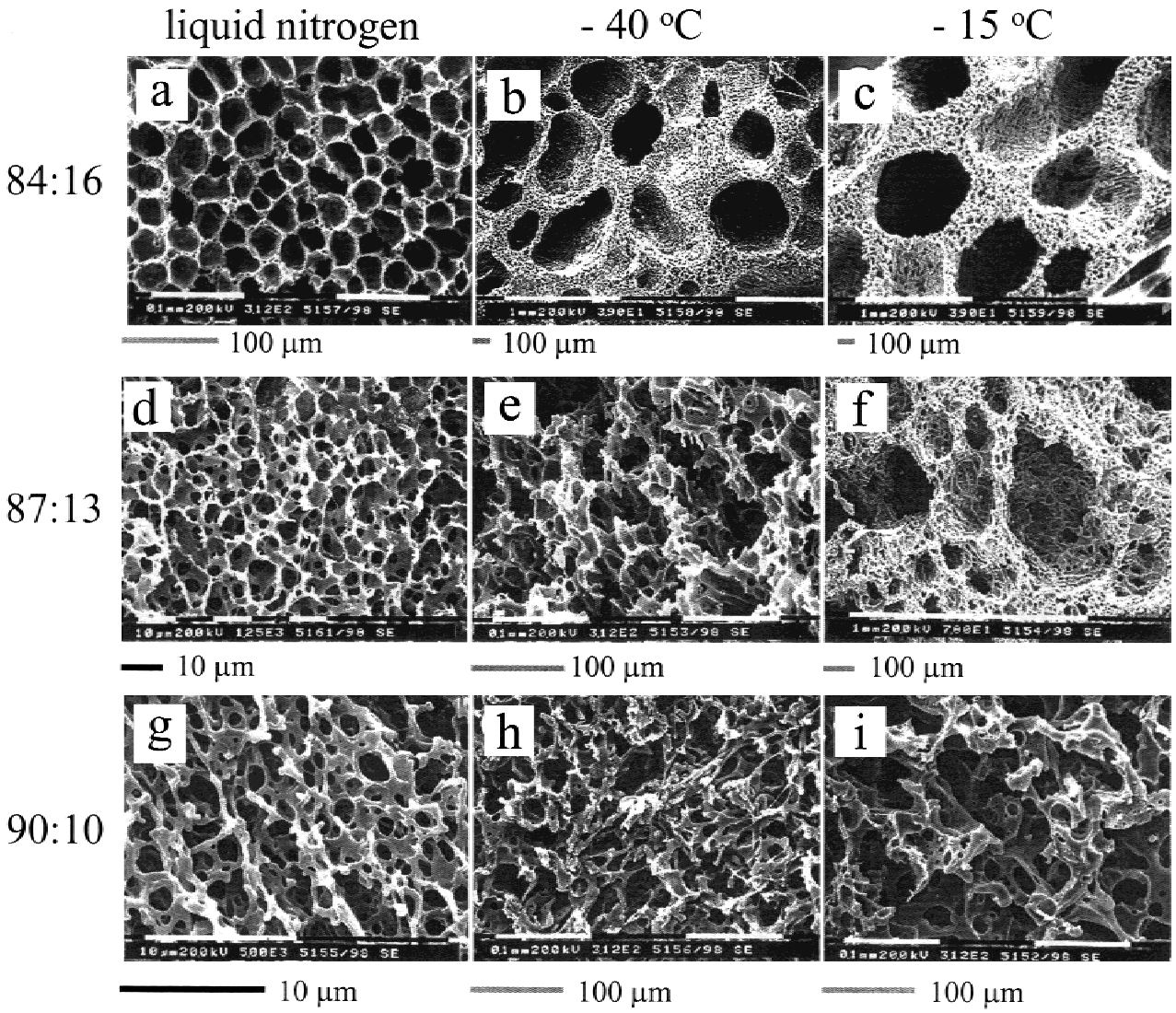

Figure 5. SEM images of the cross section of PLGA scaffolds. The scaffolds were prepared by quenching the 10% (w/v) polymer solution under the following conditions: liquid nitrogen (a,d,g); −40°C (b,e,h); −15°C (c,f,i). The volume ratios of dioxane and water were: 84/16 (a–c); 87/13 (d–f); 90/10 (g–i).

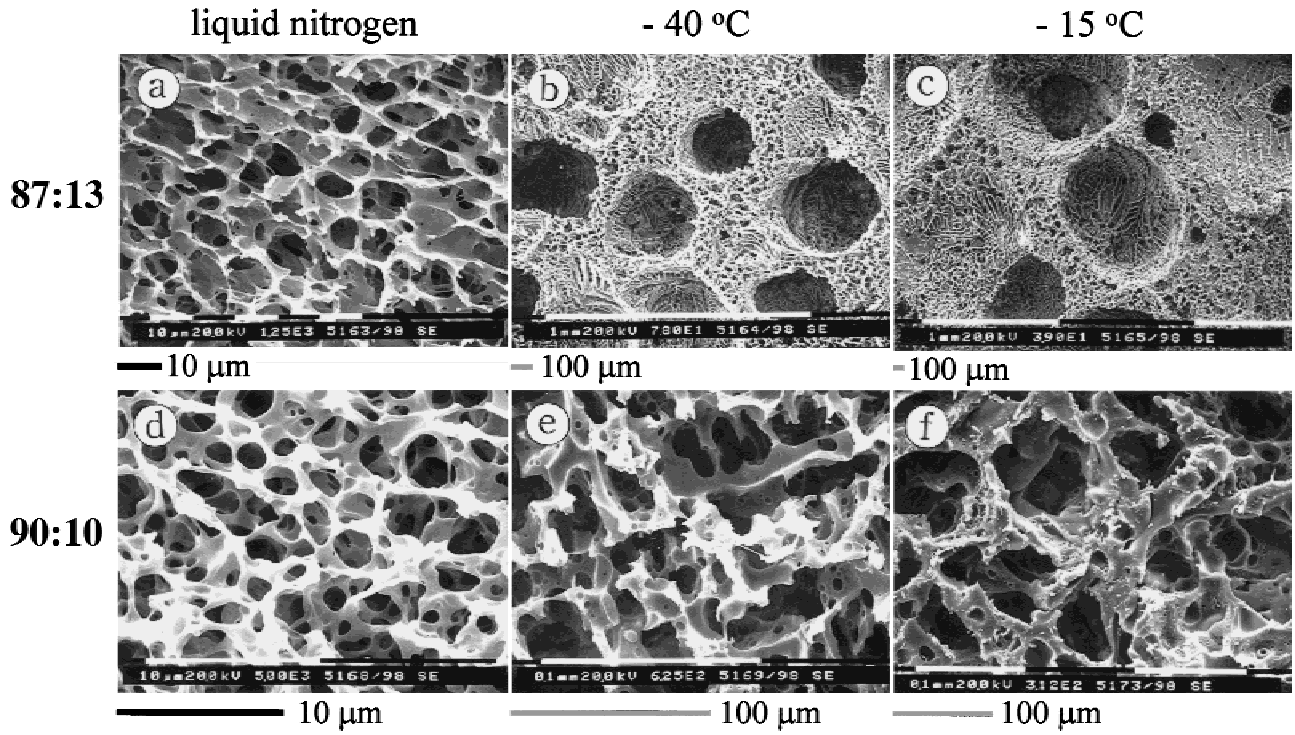

Figure 6. SEM images of the cross-section of PDLLA scaffolds. The scaffolds were prepared by quenching the 10% (w/v) polymer solution under the following conditions: liquid nitrogen (a,d); –40°C (b,e); –15°C (c,f). The volume ratios of dioxane and water were: 87/13 (a–c); 90/10 (d–f).

11.8°C, allowing easy removal by a freeze-drying method. Figure 2 shows cloud points of various polymer solutions as a function of polymer concentration. Since the molecular weights of the polymers used in this experiment were polydisperse, cloud point curves were constructed instead of the binodal curve, which is only applied to a polymer with monodisperse molecular weight.[26,27] Up to 25% (w/v) polymer concentration, the cloud point increases with increasing concentration, but does not exhibit a critical polymer concentration (ϕ_c) corresponding to an upper critical solution temperature (UCST). This means that the polymer concentration range in this study was located in the left region of ϕ_c. The cloud point curves of the PDLLA and PLGA polymer solution were almost similar, but PLLA cloud points appeared about 20°C higher than those of PDLLA or PLGA. This difference is supposed to originate from the difference in the polymer amorphous/crystallinity, since cloud points were independent of the polymer molecular weight.[21] In addition, semicrystalline PLLA polymer solution exhibited a gelation temperature curve appearing below the cloud temperature. This gelation behavior was presumed to be derived from small crystallites of PLLA, which were formed upon further slow cooling below the cloud point, and might play a role as physical crosslinks in the phase-separated polymer-rich domains.[26–28] From these different phase separation behaviors, it is conceivable that PLLA would result in different morphologies from PDLLA or PLGA scaffolds under the same experimental conditions, which will be shown later.

Figure 3 shows a series of SEM pictures for various polymeric scaffolds fabricated based on a 1% (w/v) polymer concentration in a mixture of 87/13 (v/v) dioxane/water system. They were obtained by warming the polymer solution above the cloud point temperature, subsequent rapid quenching at liquid nitrogen temperature or –15°C, annealing overnight, and then freeze-drying for 3 days. The scaffolds obtained from the liquid nitrogen quenching resulted in a stringy and threadlike structure, while those from –15°C quenching yielded a beady structure regardless of the polymer type. The resultant microporous structures, which were mechanically fragile, suggested that at 1% (w/v) polymer concentration, both quenching temperatures permitted the polymer solution to be located within a metastable region. This is because beady/stringy structures are typically produced by the nucleation and growth mechanism of the polymer-rich phase.

TABLE I
Characterization of Porous PDLLA Scaffolds by Mercury Intrusion Porosimetry

	Liquid Nitrogen	–15°C
Porosity (%)	80.4	90.3
Surface area (m^2/g)	4.35	0.79
Pore volume (cm^3/g)	4.46	7.89

Figure 4 shows SEM pictures of different morphologies of 10% (w/v) PLLA dissolved in varying volume ratios of dioxane and water at different quenching temperatures. When the polymer solution was quenched by liquid nitrogen, a microcellular pore structure was formed, suggesting that the quenched state of the polymer solution was located within the unstable region. The cell sizes in the scaffolds dramatically increased with increasing quenching temperature, while they tended to become a more closed cellular structure. This can be explained by the aforementioned coarsening effect, in which phase-separated polymer-poor (solvent-rich) droplets coalesce to form larger droplet domains in the later stage of phase separation. Since the coarsening effect is a kinetic behavior directed toward reaching the thermodynamic minimization of interfacial energy, one can manipulate the cellular structure by arresting the phase separation process. In this study, coarsening time scales were expected to vary by adopting different quenching temperatures. The coarsening time decreased with decreasing quenching temperature.

It should be mentioned that even though the liquid nitrogen–quenched scaffolds exhibited a microcellular morphology, their closed cell structures were somewhat different from the typical bicontinuous morphology produced by the spinodal decomposition mechanism, as shown in Figure 1.[20–22,25] As the clear polymer solution was cast into a brass mold equilibrated with liquid nitrogen temperature, it is conceivable that an instantaneous heat gradient, although of presumably very short duration, developed in the direction from the contacting mold surface to the core region of the quenched polymer sample. This would create different phase-separated morphologies from the center to the surface in the resultant scaffolds by exerting locally different phase-arresting time scales on the spinodal decomposition. Thus, the coarsening effect, until the final phase arresting occurred, was likely to play a critical role in the central region from the surface in those time scales. This effect was expected to be more pronounced in a large dimensional sample. From this reason, the prepared scaffolds had a more or less microporous surface skin structure, whereas the central region had a more macroporous structure because of the locally occurring coarsening effect. Accordingly, it should be noted that the term "quenching temperature" used here does not mean the tempera-

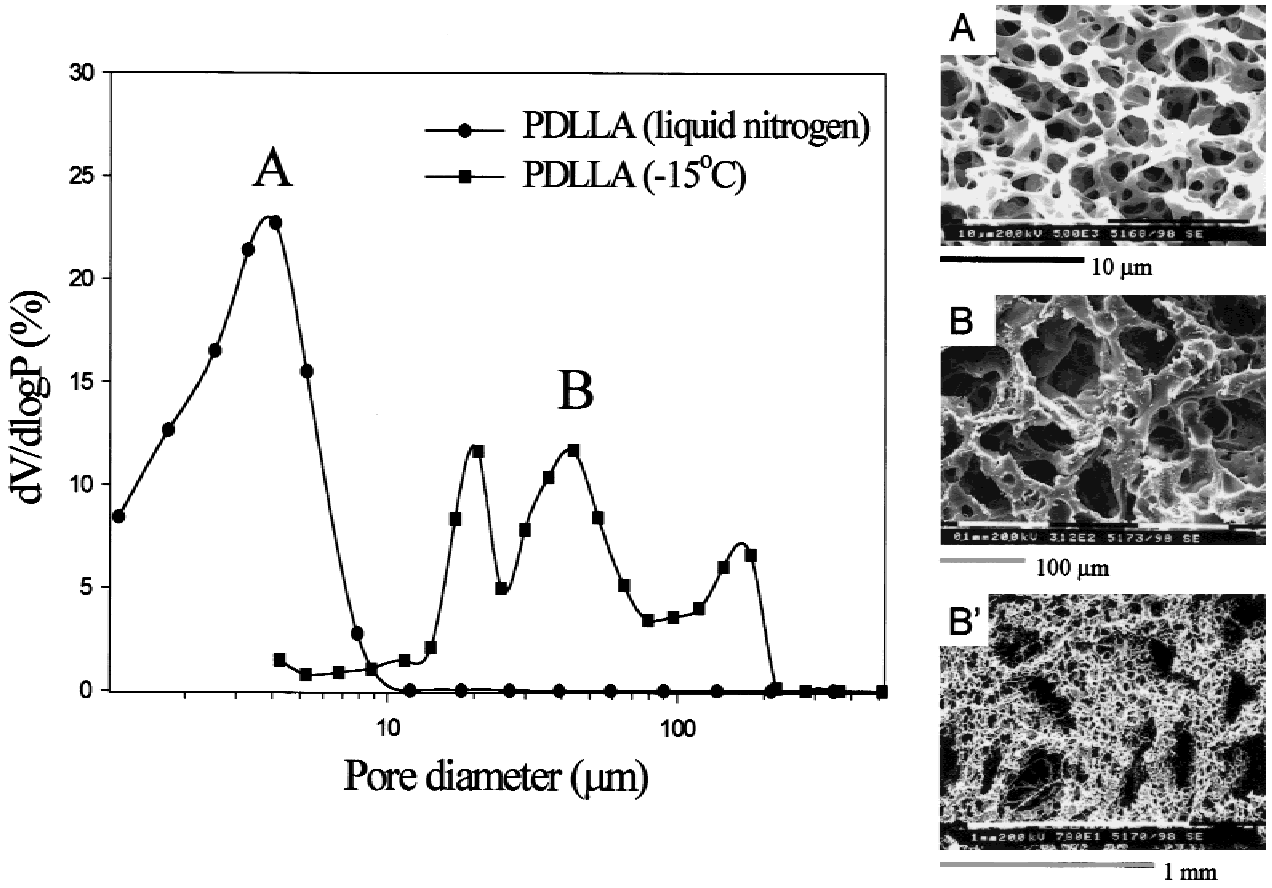

Figure 7. Pore size distribution of the PDLLA scaffolds. SEM pictures were shown to compare directly with the results determined by mercury intrusion porosimetry. (a) and (b) are the same as (d) and (f) of Figure 5, respectively. (b′) is a lower-magnification (original ×70) picture of (b).

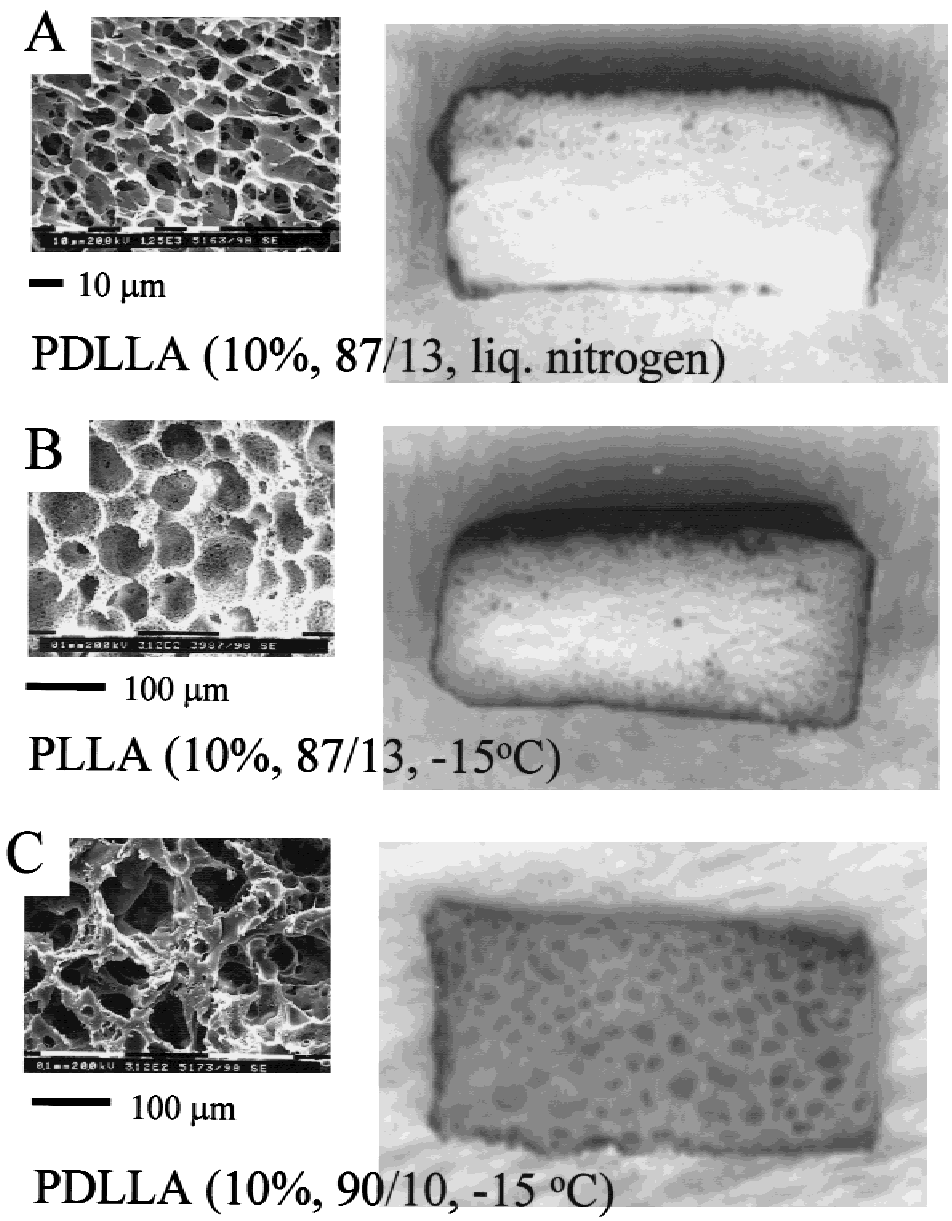

Figure 8. Cross-sectional visualization of protein distribution in the porous scaffolds. Protein was stained with a Coomassie blue dye.

ture at which the structure of phase-separated polymer solution was actually quenched, but represents only the temperature to which the samples were exposed.

The increasing amount of water in the solvent mixture tended to generate large cellular pore sizes. This was likely to be caused by the fact that as the nonsolvent volume fraction increases, a weaker polymer–diluent interaction might induce the formation of polymer poor phase with greater droplet domains.[29,30] In addition, it was reported that the gradual addition of water in a PLLA–dioxane–water system raised the cloud point curve and decreased intrinsic viscosity.[31] In our system, the cloud point curve of 87/13 (v/v) dioxane/water system was located about 15°C higher than that of 90/10 (v/v) dioxane/water system as reported.[16] Thus, the enlarged pore sizes with increasing water content were most likely due to the combined effects of weaker polymer–diluent interaction, larger quenching depth, and lowered viscosity in the cosolvent system. The PLLA scaffolds prepared using 90/10 dioxane/water did not exhibit a well-defined lacy structure different from the other two scaffolds prepared by the 84/16 and 87/13 solvent/nonsolvent compositions. This trend can be understood by the preferred occurrence of solid–liquid demixing involving crystallization, gelation, and vitrification rather than liquid–liquid demixing in the spinodal decomposition region because of enhanced interaction between polymer and diluent system.[29,30]

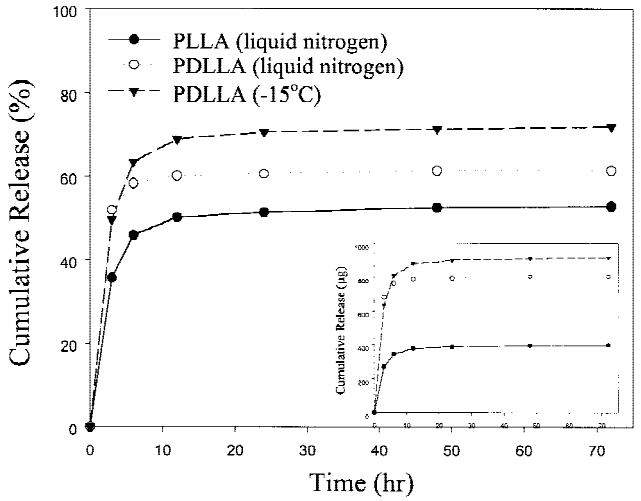

Figure 9. *In vitro* release profiles of rhGH from the porous scaffolds. (Inset) Replot of rhGH release in cumulative release amount.

Figures 5 and 6 show SEM pictures of various morphologies of PLGA and PDLLA scaffolds. Quenching at a liquid nitrogen temperature produced microporous open cellular structures as a result of the spinodal decomposition mechanism. However, increasing the quenching temperature dramatically induced the coarsening effect of pore enlargement with closing cellular structures. This effect was particularly evident for PLGA and PDLLA at 87/13 and 84/16 solvent/nonsolvent volume ratios, respectively. As the greater amount of water incorporated into the solvent mixture, closed cellular macropores up to 1000 μm in diameter could easily be seen. The PLGA and PDLLA samples at the 90/10 solvent/nonsolvent volume ratio changed their morphologies from a microporous (<10 μm) to a macroporous (~100 μm) structure while keeping an open cellular architecture. The resultant macroporous morphology seemed to be related to the coarsening effect in the later stage of liquid–liquid phase separation in a relatively viscous state plus the crystallization effect of solvent/nonsolvent mixture used. The crystallization effect, typical of solid–liquid phase separation, occurs when dioxane is used as a major solvent in the phase separation process.[16,25] The data of porosity, surface area, and pore volume for the two PDLLA scaffolds prepared by quenching at liquid nitrogen temperature and −15°C are shown in Table I. Figure 7 shows the pore size distribution of the two scaffolds as determined by mercury intrusion porosimetry. The scaffold fabricated by quenching at liquid nitrogen temperature showed a unimodal pore size distribution centered about ~3 μm, whereas the scaffold prepared at −15°C exhibits trimodal distribution centered at 20, 45, and 170 μm. The results are in good agreement with those observed in the SEM pictures. The multidistribution of pore sizes clearly suggests that the coarsening process induced by adjusting the quenching temperature played a critical role in forming a macroporous open cellular structure sufficient for cell seeding and tissue regeneration. To elucidate whether the 10% PDLLA scaffold fabricated by using 90/10 dioxane/water volume ratio had an open cellular structure, rhGH was loaded into the scaffold by an immersion-soaking method and then stained with a Coomassie blue dye for cross-sectional visualization, as shown in Figure 8. The macroporous scaffold was homogeneously stained, indicating that it truly had an open cellular structure. On the other hand, the PLLA scaffold with an apparent macroporous closed cellular structure and the PDLLA scaffold appearing to have a microporous open cellular structure, as observed in their SEM pictures, did not show evidence of protein staining in the central region of their devices. This experiment clearly indicates that they do not have macroporous open cellular structures enough for the penetration of rhGH through the pores. The loading capacity of rhGH with the open cellular scaffolds was much higher than that of the closed cellular foams, but the release profiles of rhGH from the three scaffolds were similar: an initial rapid release up to 36–52% and subsequent very slow release, as shown in Figure 9. The very slow release was likely due to rhGH adsorption onto the surface of the scaffolds with a large surface area.[32,33]

CONCLUSION

Macroporous open cellular scaffolds with pore diameters above 100 μm can be readily fabricated by judiciously using the coarsening process in the later stage of thermally induced phase separation. These biodegradable scaffolds could be potentially applied for tissue regeneration that requires effective cell seeding through the macropores in the matrix.

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