A Novel Fabrication Method of Macroporous Biodegradable Polymer Scaffolds Using Gas Foaming Salt as a Porogen Additive

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Abstract: Highly open porous biodegradable poly(L-lactic acid) [PLLA] scaffolds for tissue regeneration were fabricated by using ammonium bicarbonate as an efficient gas foaming agent as well as a particulate porogen salt. A binary mixture of PLLA-solvent gel containing dispersed ammonium bicarbonate salt particles, which became a paste state, was cast in a mold and subsequently immersed in a hot water solution to permit the evolution of ammonia and carbon dioxide within the solidifying polymer matrix. This resulted in the expansion of pores within the polymer matrix to a great extent, leading to well interconnected macroporous scaffolds having mean pore diameters of around 300–400 μm, ideal for high-density cell seeding. Rat hepatocytes seeded into the scaffolds exhibited about 95% seeding efficiency and up to 40% viability at 1 day after the seeding. The novelty of this new method is that the PLLA paste containing ammonium bicarbonate salt particles can be easily handled and molded into any shape, allowing for fabricating a wide range of temporal tissue scaffolds requiring a specific shape and geometry. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res (Appl Biomater) 53: 1–7, 2000

Keywords: biodegradable polymers; poly(L-lactic acid); scaffolds; tissue engineering; ammonium carbonate

INTRODUCTION

Biodegradable porous polymer scaffolds have been used for temporal templates for tissue regeneration. The most extensively utilized biodegradable polymers are poly(L-lactic acid) [PLLA] and its copolymers with D-lactic and glycolic acid because of their versatile biodegradability and proven biocompatibility.[1] For tissue regeneration, highly open porous polymer matrices are required for high-density cell seeding and efficient nutrient and oxygen supply to the cells cultured in the three-dimensional matrices. There have been several methods to produce highly porous biodegradable polymer scaffolds such as compressed mesh of nonwoven polyglycolic acid (PGA),[2-4] solvent casting/salt leaching,[5-6] emulsion freeze drying,[7] expansion from pressurized carbon dioxide,[8-9] phase separation,[10-12] and 3-D printing technique.[13] While the compressed PGA mesh has been popularly used for soft tissue regeneration, its mechanical strength is weak and not suitable for hard tissue regeneration.[4] The solvent casting/salt leaching out technique has been extensively utilized for cell seeding and transplantation. The biodegradable scaffolds prepared by this method often exhibited a dense surface skin layer, which hampered *in vitro* cell seeding into the scaffolds and tissue in-growth after *in vivo* implantation. Additionally, poor interconnectivity between macropores lowered seeded cell viability and resulted in nonuniform distribution of the seeded cells throughout the matrix. A combined approach of salt leaching/gas foaming based on compressed carbon dioxide gas was recently introduced to alleviate the exterior skin and pore interconnectivity problem.[9] The thermally induced phase separation (TIPS) technique has been normally used for fabricating various microcellular biodegradable foams.[10-11] More porous open cellular biodegradable scaffolds with a mean pore diameter of well over $\approx$100 μm by utilizing the coarsening effect were more recently prepared in our laboratory.[12] The coarsening effect, which enlarges the pore diameters by the tendency to reduce an interfacial free energy between phase separating domains, was induced by controlling the quenching temperature and time during the TIPS process. While the various scaffolds prepared by the above-mentioned methods demonstrated versatile scaffold morphologies, *in situ* hand shapeable or moldable polymer scaffolds fitting to the three-dimensional geometry of specific tissue defects are also highly in demand for clinical applications. Pliable biodegradable polymeric elastomers or pastes are expected to be ideal candidates for manipulating the shape, geometry, and size of the scaffolds, as long as the open

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porous structure is maintained while keeping its structural integrity.

In this study, PLLA paste containing ammonium bicarbonate particulates, acting as a gas foaming agent as well as a salt leaching porogen, were used to fabricate highly porous biodegradable scaffolds. Sodium bicarbonate salt with acidic excipients has been used for effervescent gas evolving oral tablets. Ammonium bicarbonate salt upon contact to an acidic aqueous solution and/or incubated at elevated temperature evolves gaseous ammonia and carbon dioxide by itself. Ammonium bicarbonate salt particles were incorporated into a biodegradable gel prepared by dissolving PLLA in an organic solvent. The resultant putty paste, behaving like a viscous slurry, was molded and directly dried under vacuum or incubated in a hot water solution to remove or leach out the salt particles with concomitantly generating gaseous ammonia and carbon dioxide within a solidifying polymer matrix. Morphologies of the resultant scaffolds were examined by scanning electron microscopy (SEM), and their porosity and pore volume were determined by mercury intrusion porosimetry. Primary rat hepatocytes were seeded within the scaffolds to determine cell seeding efficiency and viability after the seeding.

MATERIALS AND METHODS

Materials

Poly(L-lactic acid) (PLLA) was purchased from Polysciences, Inc. (lot #405221, Warrington, PA). Nominal weight average molecular weight was 300,000. Ammonium bicarbonate (NH_4HCO_3) and Dulbeccos's modified Eagle's medium (DMEM) with 4.5 mg/mL glucose were obtained from Sigma (St. Louis, MO), insulin from Squibb (Princeton, NJ), glucagon from Lilly (Indianapolis, IN), epidermal growth factor (EGF) from Collaborative Research (Bedford, CA), hydrocortisone from Upjohn (Kalamazoo, MI), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Dozindo (Japan). Other reagents were of analytical quality.

SCAFFOLDS PREPARATION

Vacuum Drying. PLLA polymer (1.5 g) was dissolved in 15 mL of methylene chloride under magnetic stirring and 9 g of ammonium bicarbonate (100–500 μm) was added to the solution and mixed vigorously. A disc-shaped brass mold having a dimension of 5 cm diameter and 2.5 mm thickness was used. The polymer/salt/solvent mixture paste was cast into the mold, and then air-dried under atmospheric pressure to remove methylene chloride. A gas foaming process was accomplished by drying under vacuum (9×10^{-6} Torr) for two weeks, and then residual salts were eliminated by immersing it into an excess amount of warm water (40°C). The samples were further freeze-dried for several days in order to preserve the samples for a longer period.

Gas Foaming in Hot Aqueous Solution. Highly viscous polymer solution was prepared by dissolving PLLA polymer in chloroform at a concentration of 83.3 mg/mL. Ammonium bicarbonate salt particulates (weight ratios of NH_4HCO_3 to PLLA used were 10:1 and 20:1) were added to the PLLA solution and mixed thoroughly with a spatula. Sieved ammonium bicarbonate particles in the range of 180–300 or 300–500 μm were used. The paste mixture of polymer/salt/solvent was cast into a disc-shaped Teflon mold (1 cm diameter and 1.1 mm thickness) or a cylinder-shaped mold (6 mm diameter and 12 mm height). After chloroform was partially evaporated under atmospheric pressure, the semi-solidified samples were immersed into an excess amount of hot water (90°C) until no gas bubbles were generated ($\approx$5 min). Afterwards, the samples were placed into cold water for 20 min, and then freeze-dried for several days and stored at −20°C until use.

Scaffolds Characterization

Differential scanning calorimetry (DSC, DuPont 2000) was employed to examine the thermal properties of the prepared scaffolds. Glass temperature (T_g) was measured in the first heating scan and the melting temperature (T_m) and its corresponding enthalpy (ΔH_m) were determined in the second scan. Temperature was scanned from −10– 200°C with a heating speed of 10°C/min. Mercury intrusion porosimetry (Porous Materials, Inc., NY) was used to determine the total pore volume and porosity of the scaffolds.[12] The pressure was applied up to 200 psia. Compression modulus of the scaffolds was evaluated at room temperature on an Instron 5538 equipped with a 10 N load cell. Cross-head speed was set at 2 mm/min. Cylinder-shaped samples measuring 6 mm in diameter and 12 mm in height were used, according to ASTM method F451-95.

Hepatocyte Isolation

Rat hepatocytes were isolated from 4 week-old male Sprague-Dawley rats, weighing *ca.* 125 g, using the previously described method by Dunn et al.[15] Briefly, the liver of the ether-anesthetized rat was first perfused through the portal vein *in situ* with 200 mL of perfusion buffer containing 1 mM EDTA at a flow rate of 25 mL/min. Perfusion buffer (pH 7.4) consisted of 154 mM NaCl, 5.6 mM KCl, 5.5 mM glucose, 35 mM $NaHCO_3$, and 20 mM *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (HEPES) and was saturated with oxygen by purging with 95% O_2 and 5% CO_2 at 1 day before cell isolation. Rat liver was perfused with 100 mL of 0.05% (w/v) collagenase in perfusion buffer with 5 mM $CaCl_2$. Temperature was controlled at 37°C by using a heat exchanger during the liver-perfusion. After the perfusion, the swollen liver was transferred to a petri dish containing 15 mL of ice-cold perfusion buffer. Single cells of hepatocytes were prepared by teasing the liver capsule with smooth cell-scraper, and the

TABLE I. Preparation Conditions for PLLA Scaffolds

Batch Number	Weight Ratio of NH_4HCO_3 to PLLA	Solvent	Salt Size Range (μm)
1-1	6:1	CH_2Cl_2	100–500
2-1	10:1	$CHCl_3$	180–300
2-2[a]	10:1	$CHCl_3$	180–300
2-3	20:1	$CHCl_3$	180–300
2-4	10:1	$CHCl_3$	300–500

[a] Prewetted by ethanol before gas foaming process.

resulting cell suspension was filtered through nylon mesh of 125 μm grid size (Small Parts, Miami, FL). The cells were washed twice with perfusion buffer, and viable hepatocytes were separated by centrifugation at 50 g for 15 min by using Percoll solution, containing Hanks' balanced salt solution (1 $\times$ HBSS, pH 7.4). Routinely, about 250 million cells were isolated with *ca.* 90% viability as judged by the trypan blue exclusion test.

Cell Seeding and MTT Assay

Scaffolds were prewetted in pure ethanol with an orbital shaking at 100 rpm for 2 h, and then ethanol was exchanged with an excess amount of deionized water and subsequently 33 mM phosphate buffered saline (PBS, 0.1 M NaCl, pH 7).[16] After the excess PBS was removed from the scaffolds with a sterilized dry tissue (Kimwipes), cell suspension (40 μL) with a cell density from 7.0×10^4 to 4.8×10^5 cells/device was loaded onto the scaffolds with a micro-pipette and was allowed to infiltrate into the scaffolds by placing them on sterilized dry cotton tissue. The sample was maintained at 37°C under 5% CO_2 condition for 5 min and then 400 μL of culture medium was added to the plate containing the cell-loaded scaffolds. The culture medium consisted of DMEM supplemented with 10% (v/v) FBS, a hormone mixture of 0.5 unit/mL insulin, 7 μg/mL glucagon, 20 ng/mL epidermal growth factor and 7.5 μg/mL hydrocortisone, 200 units/mL penicillin, and 200 μg/mL streptomycin. Cell seeding efficiency was determined by counting the number of cells remaining in the medium after 1 day of culture. Cell viability was determined by measuring mitochondrial dehydrogenase activity of hepatocytes. Briefly describing, 5 μL of MTT solution (5 mg/mL) was freshly added to culture well containing 100 μL of fresh medium, and incubated at 37°C and 10% CO_2 for 4 h. The intracellular formazan was solubilized using 100 μL of lysing buffer containing 45% dimethyl formamide and 10% sodium dodecyl sulfate (SDS). The absorbance of formazan produced was measured at 590 nm with a Bio-Rad microplate reader. Viable cell number was determined using a linear correlation between absorbance and hepatocyte concentration.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was employed to examine (i) the surface/cross-sectional pore morphology of freeze-dried scaffolds, and (ii) the distribution morphology of hepatocytes seeded into the scaffolds. Hepatocytes seeded into the scaffolds were fixed with 2.5% (w/v) glutaraldehyde in 1 mL of 33 mM PBS at 4°C for 90 min, and then washed in 1 mL of PBS three times for 5 min each. Cells were gradually dehydrated in a series of ethanol solutions (25, 50, 60, 70, 80, 90, 100% ethanol) for 5 min each. Samples were freeze-dried and then mounted on an aluminum stub. Gold coating was ac-

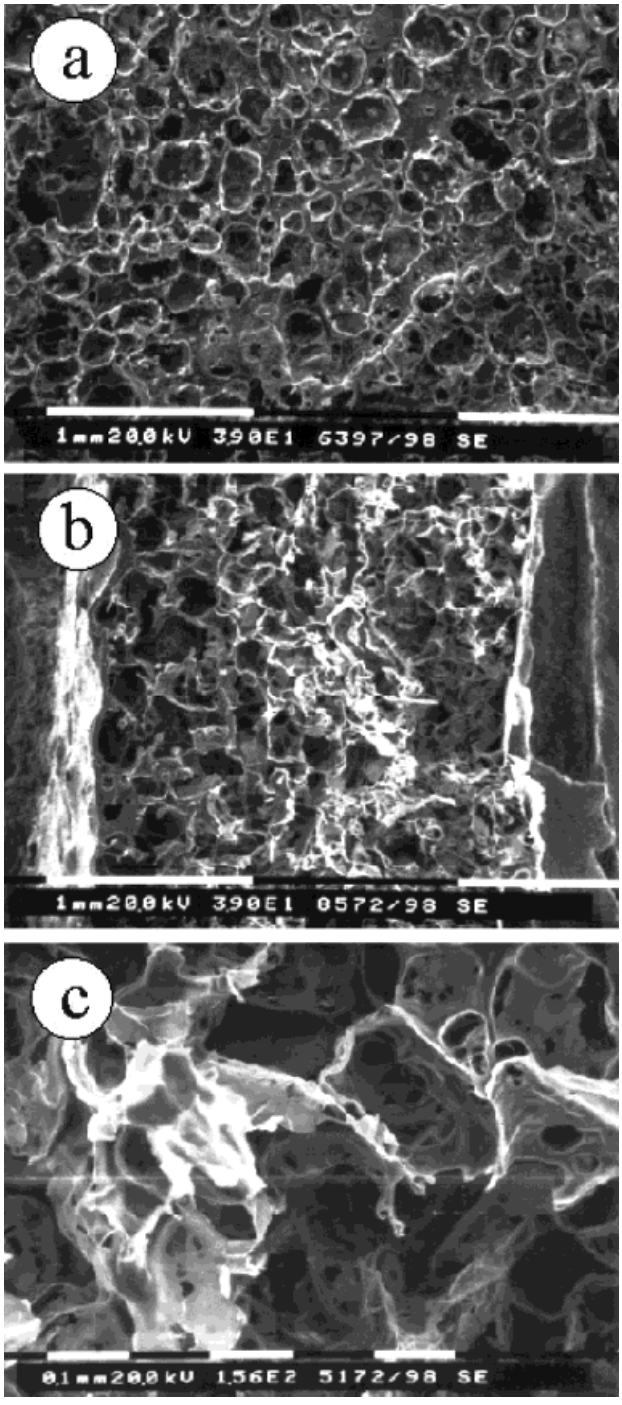

Figure 1. SEM images of surface and cross-section morphology of PLLA scaffolds prepared by vacuum drying method: (a) surface; (b) & (c) cross-section.

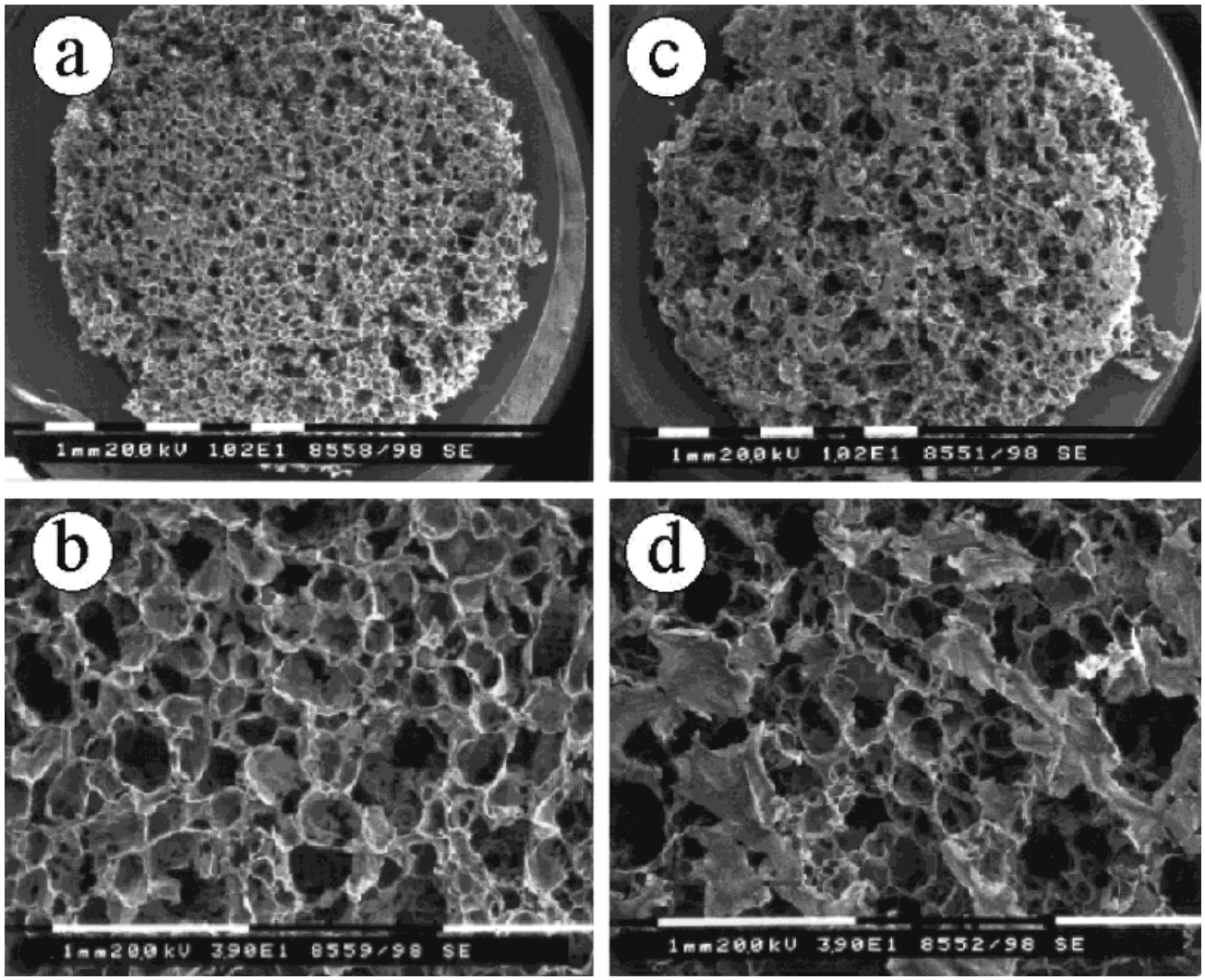

Figure 2. SEM images of surface morphology of PLLA scaffolds prepared by gas foaming in hot aqueous solution: (a) & (b) batch 2–1; (c) & (d) batch 2–4.

complished by using a sputter-coater (Hummers, Technics, USA). Ar gas pressure was set at 5 psi and the current was maintained at 10 mA for a coating time of 5 min. Visualization was carried out by using a SEM (Philips 535M).

RESULTS AND DISCUSSION

Biodegradable polymers, semi-crystalline poly(L-lactic acid) and amorphous poly(D, L-lactic-co-glycolic acid), could be made in a gel state by dissolving them in appropriate organic solvents.[14] Polymer types, molecular weight, concentration, and temperature were all important to attain the viscous gel state at room temperature. The incorporation of sieved ammonium bicarbonate salt particulates into the polymer/solvent gel phase resulted in a more malleable and hand-shapeable paste, which could be applied into molds having any shape and size. Table I lists the composition of polymer/solvent/ammonium bicarbonate for the preparation of putty pastes. The molded pastes were subjected to gas foaming of ammonia and carbon dioxide within the matrix by either direct vacuum drying or immersion in hot water.

A direct gas evolution from ammonium bicarbonate within the PLLA polymer matrix was carried out by high-vacuum drying (9×10^{-6} Torr) for two weeks at room temperature. Although most of ammonium bicarbonate salts were removed by sublimation from the matrix under this condition, subsequent salt leaching in an aqueous solution was carried out for the complete elimination of residual salts. Figure 1 shows SEM images of the surface and cross-sections of the freeze-dried scaffolds. Closed cellular macropores are observed on the surface as a trace of ammonium bicarbonate salts. The cross-section view reveals that although 200–300 μm interconnected macropores were present in the interior region, an undesirable dense skin layer was formed on the surface. The generation of a skin layer on the surface of the scaffolds was routinely observed when volatile solvents were evaporated from the interior region of the matrix to the surface. Addi-

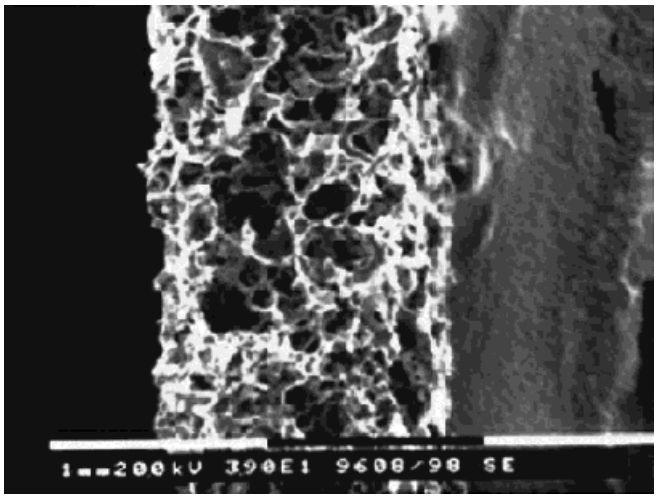

Figure 3. SEM image of cross-section morphology of PLLA scaffolds prepared by gas foaming in hot aqueous solution (batch 2–4).

tionally, the scaffolds prepared by the vacuum drying method show a partially collapsed structure, which was supposed to be caused by the slower removal process of the residual organic solvent during the vacuum drying process.

As a second approach, the molded pastes were placed in a hot water solution for simultaneous gas foaming/salt leaching. The incorporated ammonium bicarbonate particulates could be eliminated from the matrix through a gas foaming process by which they were converted to gaseous ammonia and carbon dioxide. In this case, dispersed ammonium bicarbonate particulates were also leached out with concomitantly generating gases in the solidifying blend matrix. It should be noted that it was important not to completely remove the volatile organic solvent in the solidified blend mixture before proceeding to the gas foaming process, because the polymer gas foaming process occurs more readily in an elastic and rubbery gel state rather than in a glassy state of the polymer. Figures 2 and 3 show SEM pictures of the scaffolds prepared by incorporating sieved ammonium bicarbonate salts having two different size distributions (180–300 μm and 300–500 μm) into the PLLA/chloroform gel. In contrast to the sample prepared by the vacuum drying method as shown in Figure 1, the surface structures are highly porous without showing a surface skin. Pore sizes ranging from 200–500 μm appear to be dependent on the size of the sieved ammonium bicarbonate particulates, as expected. Particularly, the uniform distribution of well interconnected pores from the surface to core region can be visualized. This macroporous open cellular structure might be generated by immediate gas evolution of salt particulates and subsequent rapid gas flow-out throughout the semi-solidified matrix to the aqueous medium. Early solidification of the polymer by rapid diffusion of residual chloroform in the blend mixture into the aqueous solution resulted in maintaining the original shape and size of the paste added in the mold, in contrast to the vacuum drying method. The cross-sectional view of the scaffold is shown in Figure 3, which clearly represents how much open porous scaffold morphology could be obtained from surface to surface.

TABLE II. Porosity and Pore Volume of PLLA Scaffolds

Batch Number	Porosity[a] (%)	Porosity[b] (%)	Pore Volume (cm^3/g)
1-1	—	86.60	7.82
2-1	90.36	88.73	9.87
2-2	92.04	89.89	12.62
2-3	95.12	93.49	19.21
2-4	93.52	91.15	11.94

[a] Determined by gross weight and volume measurements.
[b] Determined by mercury intrusion porosimetry.

Porosity and pore volume for various scaffolds were determined by a mercury intrusion porosimetry, which are listed in Table II. The porosity values were near and above 90%, proving that the ammonium bicarbonate gas foaming/salt leaching method is an excellent means for the biodegradable scaffold preparation. Porosities increased with the increase of the mixing weight ratio of salt/polymer, as expected. Porosity and pore volume results of the scaffolds prepared by vacuum drying are slightly lower than those fabricated by the aqueous solution immersion method. This was caused by the collapsed structure during the vacuum drying, as mentioned above. It should be mentioned that average pore diameters determined by the mercury intrusion porosimetry gave underestimated values of around $\approx$100 μm as opposed to the SEM observation.[7] This was because inherent limitations and assumptions, such as cylindrical pore geometry, are involved in the mercury intrusion method. Thus, direct visual estimation of the SEM image produced more reliable pore size data.

Table III lists compression modulus results for the scaffolds fabricated in a cylinder shape. The compression modulus value of the scaffold prepared by using a 10/1 ammonium bicarbonate/polymer ratio is much higher than the other scaffold due to the less porous morphology. The compression modulus result is comparable to that recently reported for the PLGA scaffold prepared by the method of salt leaching/gas foaming from compressed carbon dioxide.[9] Differential scanning calorimetry (DSC) was used to determine whether any change in polymer structure occurred during the scaffold fabrication process. Thermal properties of the scaffolds such as glass transition temperature (62.5°C), crystalline melting temperature (177.5°C), and melting enthalpy (ΔH = 34.5 J/g), were not changed at all as compared to those of raw polymer. The gas foaming/salt leaching method introduced in this study has distinctive advantages relative to other previous

TABLE III. Compression Modulus Values of Porous PLLA Scaffolds

Weight Ratio of NH_4HCO_3 to PLLA	Compression Modulus (kPa)
10 : 1	242.3 ± 32.5
20 : 1	65.8 ± 5.4

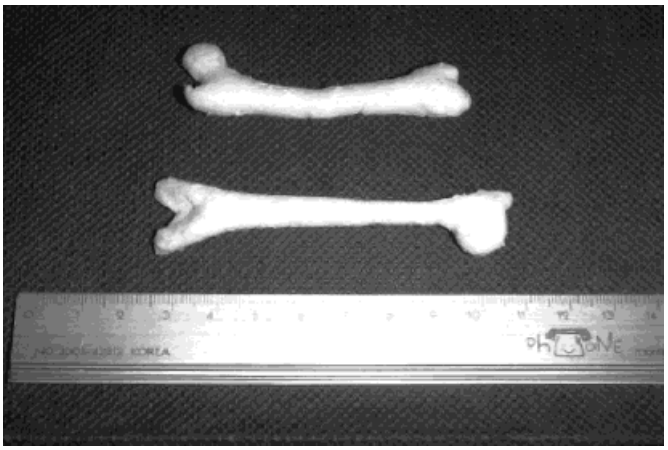

Figure 4. Photograph of a bone-shaped polymer scaffold prepared by a hand-shaping process.

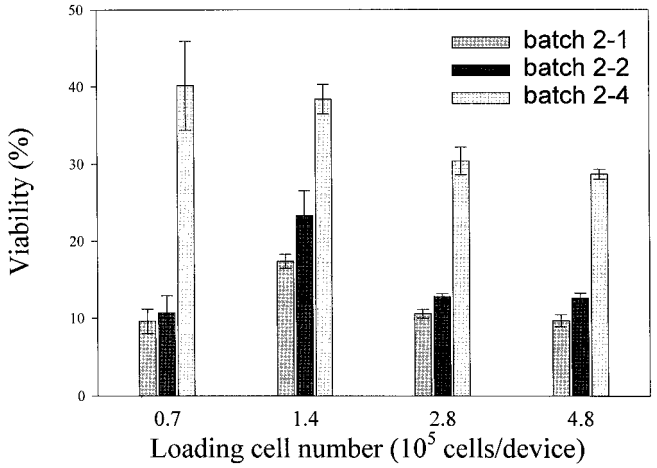

Figure 5. Viability of hepatocytes seeded in the scaffolds as a function of cell loading density at 1 day after seeding.

methods. The paste of polymer/solvent/ammonium bicarbonate blend mixture can be manipulated by hand, a surgical knife, or a spatula to make any shape and size of the scaffold. For example, Figure 4 shows the photograph of a model femoral bone fabricated by the hand shaping process.

Cell seeding efficiency for three disc-shaped scaffolds was determined using rat hepatocytes. To facilitate the cell infiltration through pores in the scaffolds, the scaffolds were prewetted by ethanol first and then incubated in PBS buffer.[16] They were placed onto a dry cotton tissue sheet. A capillary wetting force of the dry tissue that drew prefilled water in the scaffold pores effectively sucked up the overlaid cell suspensions to a great extent.[17] Table IV shows the cell seeding efficiency for the three scaffolds. Over 90% cell seeding efficiencies into the scaffolds can be seen regardless of initial cell loading density, suggesting that the fabricated scaffolds using the gas foaming/salt leaching method fully satisfied the requirements of cell transplantation templates, that is, macroporous skeletal structure as well as interconnectivity between their pores. The loaded cells within pores of the scaffolds were cultured for 1 day and were tested as to whether they maintained sufficient cell viabilities by MTT assay. Figure 5 shows cell viability as a function of cell loading density for the three scaffolds. The hepatocytes loaded into the most porous scaffold (batch 2–4), as demonstrated in Figure 2 and Table II, exhibit the highest cell viability up to 40%. The enhanced cell viability for the more porous scaffold was presumably caused by more efficient transports of oxygen and nutrients to the loaded cells in the scaffold. For the batch 2–4 scaffold, cell viability tends to decrease with increased cell loading density. This is easily understandable, if the above mass transport problem was involved in the viability of the loaded cells. The batch 2–1 and 2–2 scaffolds had the same formulation recipe (see Table I), but the 2–2 scaffold was pretreated with ethanol prior to an immersion into an aqueous solution for an efficient wetting and gas foaming. The batch 2–2 scaffold had slightly more porous structure than the batch 2–1 scaffold as listed in Table II, resulting in the slightly better cell viability. For batch 2–1 and 2–2 scaffolds, cell viability shows its maximum at 1.4×10^5 initial cell loading number. It is likely that loaded cells at this cell number were at an optimal density with regard to the mass transport efficiency of nutrients in the two less porous scaffolds. Figure 6 shows SEM pictures of hepatocytes attached onto the surface of interior pores in the batch 2–4 scaffold. The top picture shows well-adhered and spread hepatocytes onto the surface of macropores, and the bottom picture shows aggregated hepatocytes onto the surface of less open pores.[18] The accumulation and aggregation of seeded cells in some dead end pores might be more susceptible to reduction in cell viability owing to the limited mass transfer effect.

TABLE IV. Loading Efficiency of Cells Seeded into Porous PLLA Scaffolds

Initial Cell Loading Density (Cells/Device)	Loading Efficiency		
	Scaffold 2–1	Scaffold 2–2	Scaffold 2–4
0.7×10^5	98.13 ± 4.79	98.28 ± 2.83	93.97 ± 0.22
1.4×10^5	94.06 ± 0.76	94.29 ± 1.31	90.29 ± 1.31
2.8×10^5	98.53 ± 0.65	96.88 ± 1.36	97.72 ± 0.82
4.8×10^5	98.11 ± 0.44	96.88 ± 0.35	97.93 ± 0.82

CONCLUSIONS

It has been demonstrated that open porous biodegradable scaffolds can be fabricated by a new method of gas foaming/salt leaching process. A paste composed of polymer/solvents/gas foaming salt can be hand-shapeable and moldable, sufficient to make any shape and size scaffolds fitting the desired dimension of tissue defects. The resultant scaffolds have a well interconnected macroporous structure of pore size ranging from 100–500 μm. Moreover, a dense surface skin layer

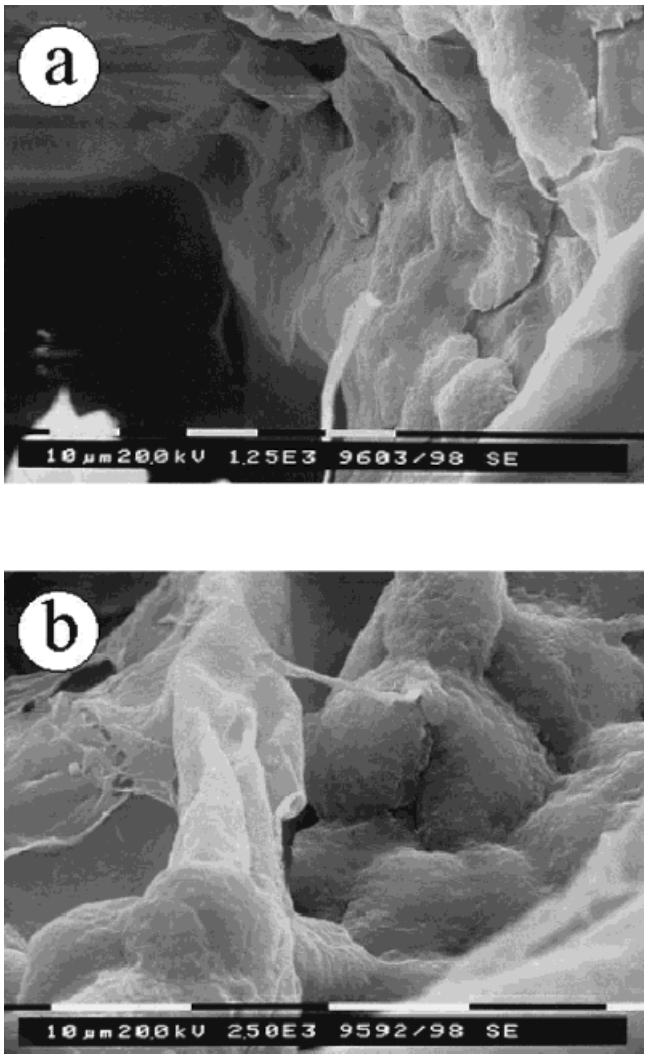

Figure 6. SEM images of hepatocytes attached onto the surface of interior pores at 1 day after seeding.

often observed in other solvent casting/salt leaching methods, was not found on both sides of the surfaces of the scaffolds. Thus, efficient cell seeding and high cell viability after the seeding can be achieved. Since a whole scaffold fabrication process can be accomplished in a clinical setting within a short time of less than 1 h without a further freeze drying process, this simple and cost-effective method might have a wide array of potential applications for tissue regeneration in the future.

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