Novel Porous Gelatin Scaffolds by Overrun/Particle Leaching Process for Tissue Engineering Applications

Hyun Goo Kang,[1] So Yeon Kim,[2] Young Moo Lee[1]

[1] School of Chemical Engineering, College of Engineering, Hanyang University, Seoul 133–791, South Korea

[2] Nanomaterials Application Division, Korea Institute of Ceramic Engineering and Technology, Seoul 153–801, South Korea

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Abstract: Porous gelatin scaffolds were prepared using a modified overrun process, which is a novel method for preparing a porous matrix by injecting air and mixing polymer solution at low temperature. The pores in the scaffolds formed by the overrun process exhibited a dual-pore structure due to the injection of air bubbles and ice recrystallization. However, the morphology of the overrun-processed gelatin scaffolds had closed pore structures. The closed pore structure was reformed into a uniformly distributed and interconnected open structure by the combination of the overrun process and a particle-leaching technique (NaCl and sucrose). The mechanical strength and biodegradation rate of gelatin scaffolds were controlled by the matrix porosity and concentration of gelatin solution. Despite higher porosity, overrun processed gelatin scaffolds showed similar mechanical strength to freeze-dried scaffolds. After 1 week of *in vitro* culturing, the fibroblasts in overrun-processed scaffolds were widely distributed on the surface of the scaffold pores, whereas cells seeded in freeze-dried scaffolds were mainly placed on the top and bottom of the scaffolds. Therefore, the overrun process combined with a particle-leaching technique can be applied to fabricate porous scaffolds with a desirable cellular structure for tissue engineering applications. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 79B: 388–397, 2006

Keywords: scaffolds; tissue engineering; porosity; overrun process; gelatin

INTRODUCTION

The extracellular matrix (ECM) is a complex mixture of structural and functional proteins, glycoproteins, and proteoglycans arranged in a unique, tissue specific three-dimensional structure.[1–2] The role that the ECM plays is far greater than just providing a physical support for the cells. It provides a substrate containing adhesion proteins required for cell adherence, and regulates cellular growth and function by presenting different kinds of growth factors to the cells.[1–2] An ideal scaffold should be designed by mimicking the native ECM as much as possible, both chemically and physically. Scaffolds for tissue regeneration need to be three-dimensional and highly porous to support uniform cell attachment, proliferation, and ECM production and need to have an interconnected and permeable pore network to promote nutrient and waste exchange.[3–5] Pore size plays a role in tissue growth by providing an internal surface area available for cell attachment, spreading, and expansion.[3–6] Therefore, scaffold processing techniques used to date have focused on the development of porous materials via gas formation,[7–9] electrospinning,[10–12] three-dimensional printing,[13] phase separation,[14–16] freeze-drying,[17,18] and solvent casting/particle leaching.[19–21]

In this study, novel porous scaffolds were prepared using the overrun process, which has been mainly applied to the manufacturing of soft ice cream.[22–24] The overrun process is valuable for entrapping injected air during the manufacturing process at a low temperature because the entrapped air produces the scaffold pores.[23,24] In particular, the overrun process can easily control the porous structure (pore size and distribution) of matrices by adjusting factors, such as impeller rate, operative temperature, and concentration of polymer solution.[22]

Although synthetic biodegradable polymers are now widely used in tissue engineering because of their generally good strength and adjustable degradation rate, chemically and biologically inert polymeric materials are unlikely to induce cell adhesion and tissue formation. To overcome this drawback of the synthetic materials, naturally occurring polymers extracted from the native ECM such as collagen have been widely used to modify the synthetic material to improve cell adhesion properties.[1]

Therefore, we prepared overrun-processed porous scaffolds composed of gelatin as a model polymer, which is a

Correspondence to: Y. M. Lee (e-mail: ymlee@hanyang.ac.kr)

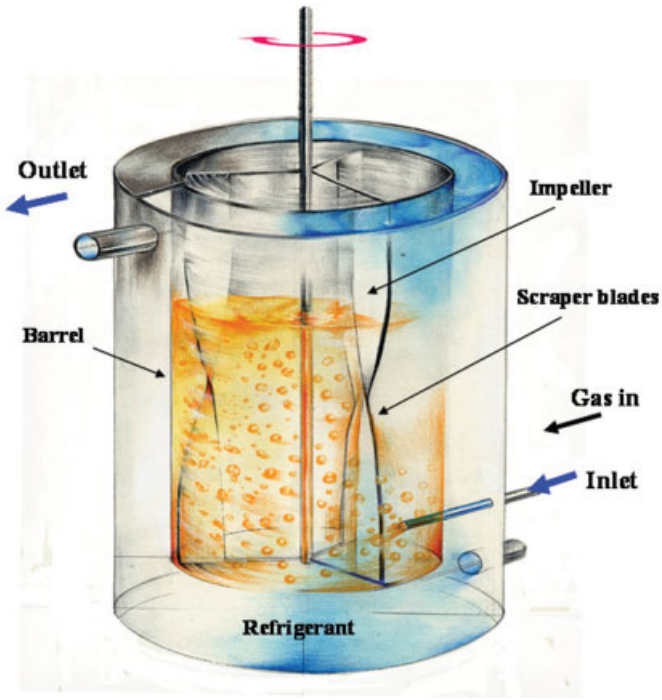

Figure 1. Schematic diagram of the apparatus used in the overrun process system. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

natural polymer obtained by a controlled hydrolysis of collagen, and is the major constituent of skin, bones, and connective tissues.[25] Gelatin is composed of a unique sequence of amino acids and contains repeating sequences of glycine-X-Y triplets, where X and Y are usually proline and hydroxyproline.[25,26] These sequences are responsible for the triple helical structure of gelatin and its ability to form gels by immobilizing water where helical regions form in the gelatin protein chains. Thus, gelatin easily forms gels by changing the temperature of its solution. This technique has been used in many tissue engineering applications because of its biocompatibility and ease of gelation.[25–31] In addition, the scaffolds prepared by an overrun/particle leaching process were compared with conventional freeze-dried scaffolds in morphology, mechanical strength, biodegradation, and *in vitro* cellular ingrowth.

MATERIALS AND METHODS

Materials

The gelatin used was derived from bovine skin, and was purchased from Sigma Chemical (St. Louis, MO). 1-ethyl-(3–3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), α-D-glucopyranosyl-β-D-fructo-furanoside (sucrose), and type IV collagenase (388 units/mg digestion activity) were also purchased from Sigma Chemical. Sodium Chloride (NaCl) was purchased from the Daejung Chemicals & Metals (Korea). Human fibroblasts were aseptically isolated from a foreskin donated by the Urology Department of Hanyang University Hospital in Seoul, Korea. Dermal fibroblast culture medium was composed of Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Rockville, MD) and 10% fetal bovine serum (FBS, Gibco BRL). Dulbecco's phosphate buffered saline (PBS), trypsin-EDTA (0.05% trypsin 5.3 m*M* EDTA·4 Na), and penicillin-streptomycin (100 U/mL) were also purchased from Gibco BRL. Trypan blue (0.4%) T-8154 and sodium bicarbonate were purchased from Sigma. All

TABLE I. Composition and Characteristics of Scaffolds Prepared by the Freeze-Drying and Overrun Process

Scaffold type	Sample code	Added particle ratio (wt %)[a] NaCl	Added particle ratio (wt %)[a] Sucrose	Conc. of gelatin (wt %)	Porosity (%)[b]	Tensile strength[c] (KPa)	Elongation at break[c] (%)
Freeze-drying process	FD1	–	–	1	74.08 ± 0.9	13.91 ± 0.42	11.14 ± 0.31.
	FD5	–	–	5	52.07 ± 1.5	41.23 ± 0.61	8.43 ± 0.11
	FD10	–	–	10	34.69 ± 1.3	76.05 ± 2.15	5.33 ± 0.12
Overrun process	OV1	–	–	1	80.18 ± 1.4	9.41 ± 0.20	11.51 ± 0.24
	OV5	–	–	5	76.23 ± 1.2	72.28 ± 2.43	8.69 ± 0.12
	OV10	–	–	10	58.23 ± 0.8	87.45 ± 3.50	6.27 ± 0.08
Overrun/particle leaching process	OV1-NaCl	5	–	1	85.28 ± 0.7	8.95 ± 1.15	11.28 ± 3.01
	OV5-NaCl	5	–	5	80.73 ± 1.2	23.27 ± 0.94	10.31 ± 1.24
	OV10-NaCl	5	–	10	74.34 ± 0.4	34.76 ± 5.02	9.28 ± 1.35
	OV1-sucrose	–	5	1	84.17 ± 1.5	9.17 ± 3.10	11.05 ± 1.21
	OV5-sucrose	–	5	5	78.43 ± 1.7	31.63 ± 1.35	9.87 ± 0.65
	OV10-sucrose	–	5	10	70.43 ± 1.8	60.57 ± 4.97	5.84 ± 0.14

[a] (Weight of particle/weight of gelatin) × 100.
[b] The porosity of gelatin scaffold sample (1.0 × 3.0 × 0.2 cm^3) were determined by mercury porosimeter (Autopore II 9220, Micrometrics) (n = 3).
[c] Mechanical properties of gelatin scaffolds (1.5× 4.0 × 0.3 cm^3) were measured by a universal testing machine (UTM, INSTRON No.4465) (n = 3).

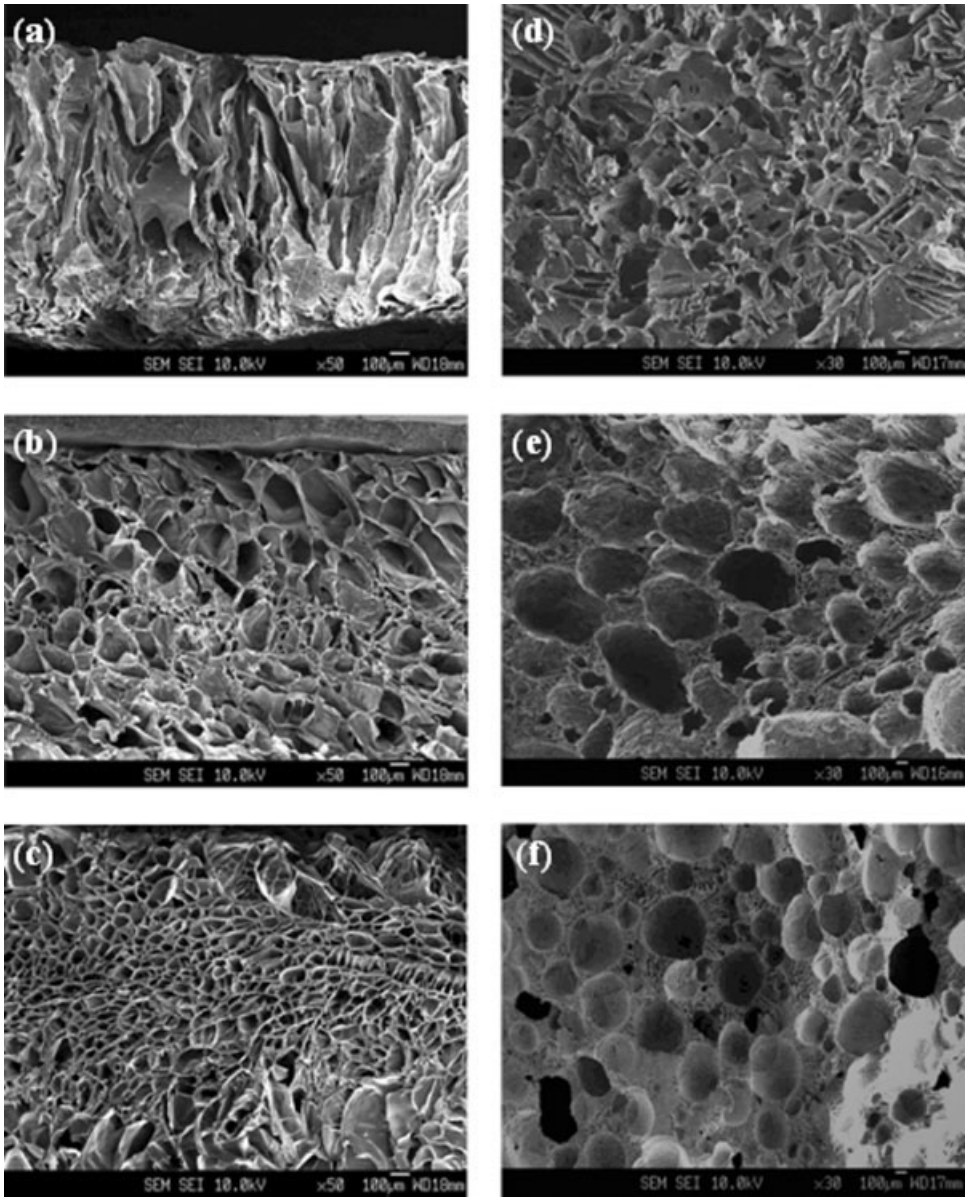

Figure 2. Cross-sectional morphologies of gelatin scaffolds prepared by the freeze-drying method and overrun process with various concentrations of gelatin solution: (a) FD1, (b) FD5, (c) FD10, (d) OV1, (e) OV5, and (f) OV10.

water was distilled and deionized with a Milli-Q Plus system (Millipore, Billerica, MA). All other chemicals used were of reagent grade and used as purchased without additional purification.

Preparation of Porous Gelatin Scaffolds by Overrun Process

Porous gelatin scaffolds were prepared by an overrun/particle leaching method using equipment composed of a refrigerated barrel, rotating impeller, and scraper blades as shown in Figure 1.

Gelatin was dissolved in deionized water in concentrations of 1, 5, and 10 wt % at 80°C for 8 h. Sodium chloride or sucrose particles were then added at 5 wt % into the gelatin solution, as listed in Table I, and homogeneously mixed. The gelatin solutions were poured into the overrun-processed chamber, which was maintained at 20°C. Low preparation temperature is chiefly used to entrap the air in solution during the overrun process of ice cream manufacture. However, in this study, gelatin scaffolds were processed at a relatively high temperature, 20°C, since gelatin has a gelling ability. Nitrogen gas (gas flow rate, 20 mL/min) was injected into the gelatin solution through the bottom of the barrel for 1 h. At a steady state, the injected gas bubbles were homogeneously dispersed into the gelatin solution by an impeller rotating[23] at 150 rpm. After a gel-like solution was formed, the solution with gas bubbles was poured into a polystyrene petri dish and frozen at −76°C for 1 day, and then lyophilized. In addition,

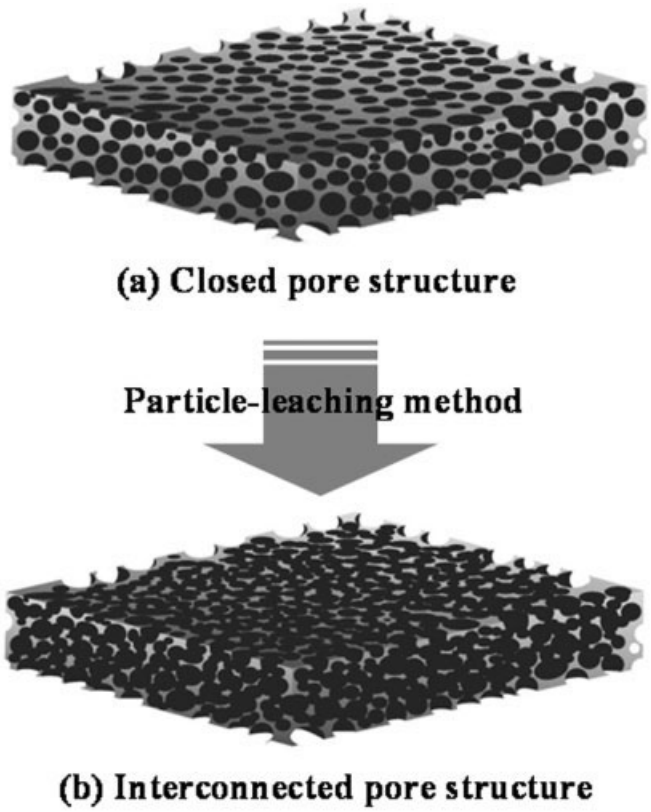

Figure 3. Schematic illustrations of the inner porous structure of scaffolds: (a) closed pore structure produced by the overrun process and (b) interconnected pore structure produced by the overrun/particle (NaCl or sucrose) leaching process.

porous gelatin scaffolds were prepared by only using the overrun process without a particle leaching step.

The dried scaffolds were crosslinked by immersion in 20 mL of an acetone:water mixture (9:1 by volume) containing 30% EDC of gelatin weight, and slowly shaken at room temperature for 1 day. The crosslinked scaffolds were rinsed with deionized water five times to remove any residual chemicals. The rinsed scaffolds were frozen at −76°C and lyophilized. The scaffold samples were then sterilized with ethylene oxide gas for cell culture.

Preparation of Gelatin Scaffold by Freeze-Drying Method

Gelatin was dissolved in deionized water in concentrations of 1, 5, and 10 wt % at 80°C for 8 h, as listed in Table I. Each solution was poured into a separate polystyrene petri dish, frozen at −76°C for 1 day, and then lyophilized. The following steps such as crosslinking, lyophilization, and sterilization were performed with the same protocols used in the overrun process.

As shown in Table I, we used the following nomenclature to define the various gelatin scaffolds, preparation method (OV; overrun, FD; freeze-drying)-the concentration of gelatin solution-particle leaching process. For example, OV5-NaCl refers to gelatin scaffolds prepared by overrun and NaCl particle leaching process with 5 wt % gelatin solution.

Morphologies of Gelatin Scaffolds

The pore morphologies of the gelatin scaffolds were investigated using a field emission scanning electron microscope (SEM, JEOL-6340F, Kyoto, Japan) at an operation voltage of 10 kV. Specimens were placed on a Cu mount and coated using a platinum-coating apparatus. The microstructures of the scaffolds were evaluated by geometrical measurement of the SEM images.

The porosity of the scaffolds was evaluated with a mercury porosimeter (Autopore II 9220, Micrometrics, Norcross, GA). Gelatin scaffolds with 0.2 mm in thickness were cut into 1.0×3.0 cm^2 rectangular shape, and weighed. Sample ($n = 3$) was placed in a cup of the penetrometer, which was closed by tightening the cap. With increasing pressure from ~3.4 to 414 kPa, the mercury penetrated through open pores of the scaffolds. By measuring the quantity of the sample pores and the equilibrium pressure at which intrusion occurs, experimental data of the pore volume distribution as a function of their diameter were obtained using the Washburn equation[32,33]:

$$D = -\frac{4\gamma\cos\theta}{P} \quad (1)$$

where D is the pore diameter, γ is the mercury surface tension taken as 480 dyne cm^{-1}, θ is the contact angle, and P is the applied pressure required to force mercury into a pore of entry diameter.

Mechanical Property

A universal testing machine (UTM, INSTRON No.4465, NY) was used to determine the mechanical strength of the gelatin scaffolds in the dry state at 25°C. The specimens had a width of 15.0 mm, a length of 40.0 mm, and a thickness of 3.0 mm. Sand paper was used to adhere on the two surfaces of gripping units, which prevent the slip of specimen during the testing. The tensile deformation rate was 1 mm/min, and the load was applied until ultimate fracture of specimen. The tensile strength was determined as the maximum point of the force-strain curve. Three specimens were tested for each sample. The mechanical strength of scaffolds was determined depending on the preparation method, concentration of polymer solution, and particle-leaching step.

Enzymatic Degradation

Enzymatic degradation of gelatin scaffolds was investigated by monitoring the percent mass loss of a sample as a function of time. The degradation behavior of the hydrogel depending on the preparation method, concentration of gelatin solution, and particle leaching process was investigated. Gelatin scaffolds ($1.5 \times 1.5 \times 0.3$ cm^3) were placed at pH 7.4 in PBS with 60 μg/mL collagenase in a shaking water bath at 37°C.

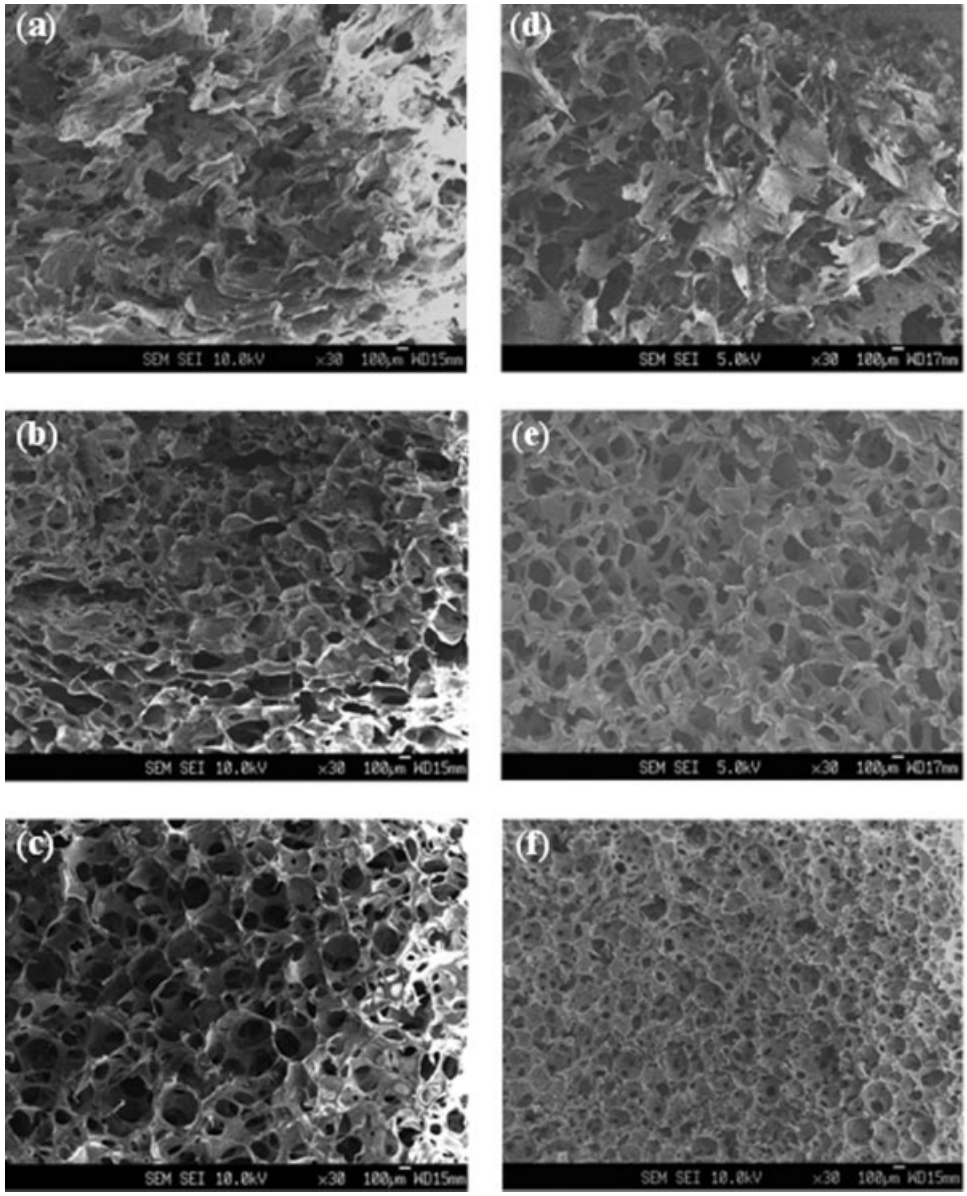

Figure 4. Cross-sectional morphologies of overrun/particle leaching processed gelatin scaffolds prepared with 1, 5, and 10 wt % gelatin solution: (a) OV1-NaCl, (b) OV5-NaCl, (c) OV10-NaCl, (d) OV1-sucrose, (e) OV5-sucrose, and (f) OV10-sucrose.

At a predetermined time, the scaffolds were removed from degradation media containing collagenase and washed three times with deionized water. The remaining weight of the scaffold was measured after lyophilization. The percent weight remaining was calculated according to the following equation:

$$\text{Remaining weight } (\%) = W_t/W_0 \times 100 \qquad (2)$$

where W_o is the initial scaffold weight and W_t is the weight of the scaffold after different time intervals in degradation media.

In Vitro Cell Culture

Gelatin scaffolds were sterilized by exposure to ethylene oxide gas. Human fibroblasts were subcultured in DMEM supplemented with 10% (v/v) FBS. The cells were harvested using 0.05% trypsin-EDTA after washing with PBS. The cultured fibroblasts were suspended in DMEM medium containing 10% FBS and seeded at a concentration of 1×10^6 cells/cm^2 on each sterilized scaffold ($1.5 \times 1.5 \times 0.3$ cm^3). Fresh medium was added to each culture dish prior to incubation at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed every 2 days for 7 days. Cultured fibroblasts in the porous gelatin scaffolds were fixed using a 10% formaldehyde solution at room temperature for 2 h. After three rinses with PBS, the scaffolds were dehydrated using a graded ethanol series comprised of 50, 70, 80, 90, and 100% ethanol for 10 min at each step. After drying, scaffold samples were embedded in paraffin and cut into 4-μm-thick sections. For histological analysis, specimens were stained

with hematoxylin–eosin (H&E) and Masson's trichrome and then visualized with a microscope.

Statistical Analysis

Statistical analysis for porosity data were described in text. Analysis of variance (ANOVA) and Newman-Keuls post-hoc analyses were performed using StatSoft Statistica 5.0 (Tulsa, CA). Statistical significance was determined at a value of $p < 0.05$.

RESULTS AND DISCUSSION

Preparation of Gelatin Scaffolds Using Overrun Process

We have prepared gelatin and gelatin-based scaffolds and studied their biological behaviors.[25–30] The freeze-drying method is usually used to prepare gelatin scaffolds because gelatin is a water-soluble natural polysaccharide. However, the gelatin-based 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. The rapid, uncontrolled quench-freezing process typically used in fabricating porous scaffolds via freeze-drying results in space- and time-variable heat transfer through the suspension, leading to nonuniform nucleation and growth of ice crystals and ultimately yielding scaffold heterogeneity.[17] On the other hand, overrun-processed porous matrices possess homogeneous morphology and dual pore structure caused by gas bubbles and recrystallization of ice crystals as reported in our previous study.[22]

The polymer solution was cooled below its freezing temperature in the barrel, resulting in the nucleation of small ice crystals. The ice crystals formed near the barrel wall were subsequently dispersed into the center of the barrel by the scraper blades. Furthermore, as the temperature drops, the ice crystal content and viscosity increases. Thus, the increased viscosity of the solution prevents the injected gas bubbles from being lost. In the case of gelatin, however, it easily forms gels by changing the temperature of its solution.[31] The viscosity of the gelatin solution is higher than other polymer solutions at room temperature. Therefore, it is difficult to form pores by gas bubbles and recrystallization of ice crystals at low temperature such as 0°C and below. Consequently, gelatin scaffolds were produced using an overrun process maintained at 20°C.

In most cases, the overrun process should be performed at low temperature which is around melting point of solvent used to entrap air bubbles in polymer solution. Thus, polymers which are soluble in only organic solvent are limited for application of overrun process. However, the limitation could be removed by using polymers with high molecular weight because of their viscosity effect.

Morphologies of Gelatin Scaffolds

Figure 2 shows the morphologies of gelatin scaffolds prepared by freeze-drying and overrun process with various concentrations of gelatin solution (1, 5, and 10 wt %). Figure 2(a–c) demonstrate the cross-sectional structure of a gelatin scaffold prepared by the freeze-drying method. Gelatin scaffolds prepared with the low concentration (1 wt %) of gelatin solution appeared as an interconnected network pore configuration characterized by a membrane-like pore structure with vertical channels [Figure 2(a)]. Also, the conventional freeze-dried gelatin scaffold formed only small pores as a result of ice crystal agglomeration [Figure 2(b,c)]. As shown in Figure 2(d,e), overrun-processed gelatin scaffolds had dual pore structures caused by gas bubbles and ice crystals except the scaffold sample prepared from 1 wt % of gelatin solution [Figure 2(d)]. The overrun-processed scaffolds with 1 wt % solution had only partially interconnected small pores, since gas bubbles could not be sufficiently entrapped because of the low concentration of gelatin solution. As the gelatin concentration increased (5 and 10 wt %), scaffolds formed a dual pore structure composed of the ice recrystalline zone between

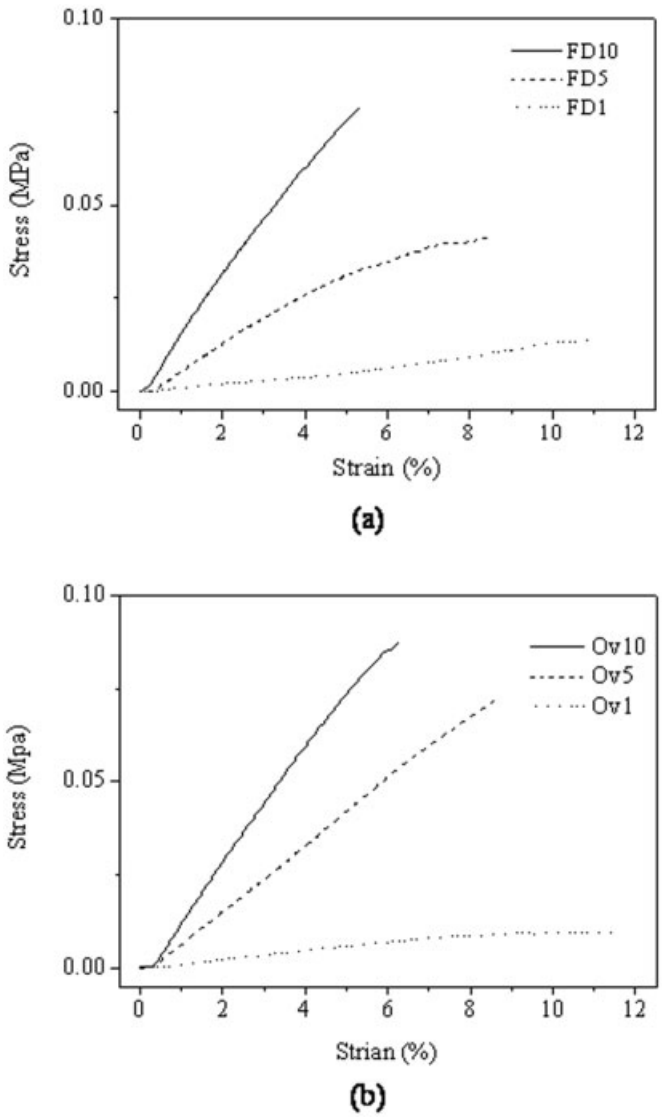

Figure 5. Mechanical strength of gelatin scaffolds prepared by (a) freeze-drying method and (b) overrun process.

gas bubbles. Ice crystals in gelatin scaffolds occurred when the gelatin solution was frozen at −76°C for 1 day after the overrun-process. As shown in Table I, the scaffold porosity gradually decreased with increasing gelatin content ($p < 0.05$). In addition, overrun-processed scaffolds showed higher porosity than freeze-dried samples prepared with the same concentration of gelatin solution ($p < 0.05$).

An ideal scaffold is a three-dimensional and highly porous one with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste.[3–6] However, the overrun-processed scaffolds still had a closed cellular structure, which was not interconnected. Poor interconnectivity between macropores lowered seeded cell viability and resulted in a nonuniform distribution of seeded cells throughout the matrix.

Therefore, we combined the particle leaching technique based on the overrun process to improve matrix interconnectivity. Based on morphologic observation, Figure 3 illustrates a hypothesis of the inner porous structure of overrun-processed scaffolds. Overrun-processed gelatin scaffolds form a closed pore structure at the proper impeller rate, operative temperature, and polymer solution, as shown in Figure 3(a).[22] The combined approach of overrun process and particle-leaching techniques could increase the pore connectivity of the scaffolds. If particles such as sucrose or sodium chloride as porogens are added to gelatin solutions, they would form inner pores between pores induced by gas bubbles and ice crystals, resulting in the formation of an interconnected pore structure as shown in Figure 3(b).

Figure 4 demonstrates the cross-sectional structure of gelatin scaffold prepared by the overrun process and particle-leaching technique using NaCl or sucrose as a porogen. Figure 4(a–c) show gelatin scaffolds prepared by mixing 5 wt % of NaCl and various concentration of gelatin solution, while Figure 4(d–f) show samples prepared by mixing 5 wt % sucrose. The scaffolds had highly porous and interconnected pore structures when particles were added. The results of porosity measurement also indicated that the additional particle-leaching process enhanced the scaffold porosity (Table I).

However, the scaffold samples prepared with 1 wt % gelatin solution (OV1-NaCl and OV1-sucrose) had an aggregated pore structure as observed in the freeze-dried scaffolds shown in Figure 4(a,b). This could result from ice crystals being a major pore component rather than injected gas bubbles due to the low concentration of gelatin solution. However, scaffolds prepared from higher concentrations of gelatin solution (5 and 10 wt %) had interconnected pore structures, and did not show an aggregated pore structure [Figure 4(b,c) and 4(e,f)]. This finding could be attributed to the fact that an increase in gelatin concentration increased the capacity for entrapment of gas bubbles and particle-induced pores in the matrix. In addition, NaCl and sucrose used as porogens produced similar porosities at equal polymer concentrations (Table I).

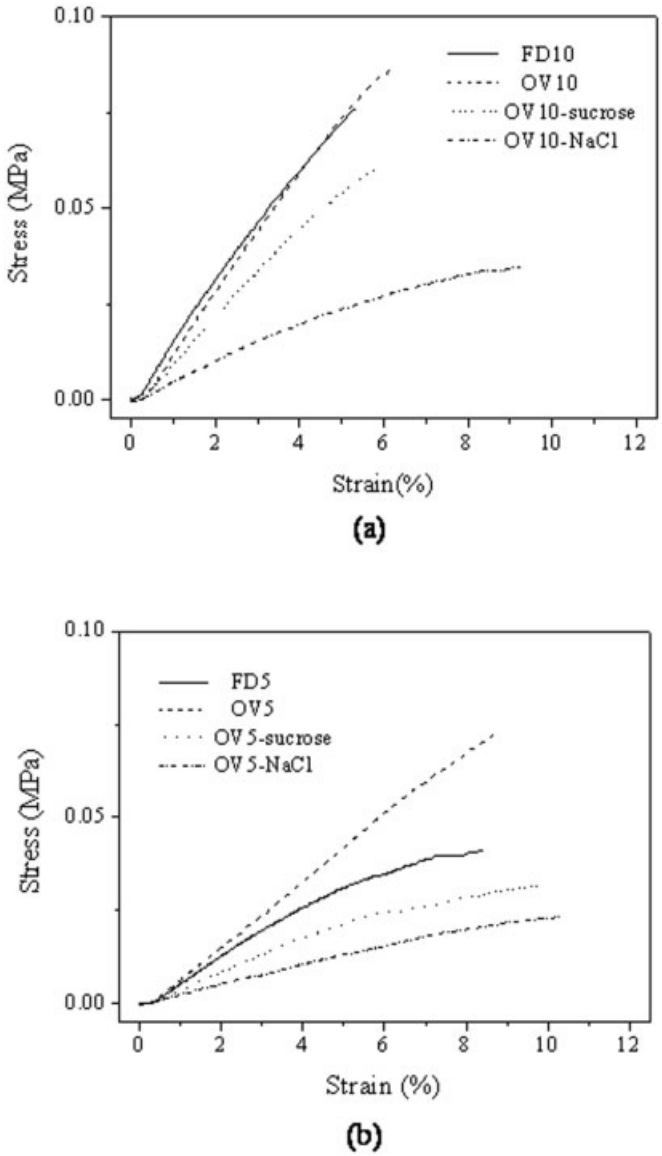

Figure 6. Mechanical strength of gelatin scaffolds depending on the preparation method: (a) 10 wt % gelatin solution and (b) 5 wt % gelatin solution.

Mechanical Properties of Gelatin Scaffolds

Figure 5(a,b) shows the representative strain–stress curves of freeze-dried and overrun-processed gelatin scaffolds, respectively. Table I summarizes the tensile strengths and elongation at break of gelatin scaffolds. The initial modulus of scaffolds gradually increased with increasing concentrations of gelatin solution for both freeze-dried and overrun-processed samples. This result may be due to the porosity of gelatin matrix. As shown in Table I, the increase in scaffold porosity caused a decrease in the tensile strength of scaffolds ($p < 0.05$).

Figure 6 indicates the strain–stress curves of gelatin scaffolds prepared by freeze-drying, overrun, and overrun/particle leaching process. Despite higher porosity, the 10 wt % overrun-processed gelatin scaffolds (OV10 sample) had a

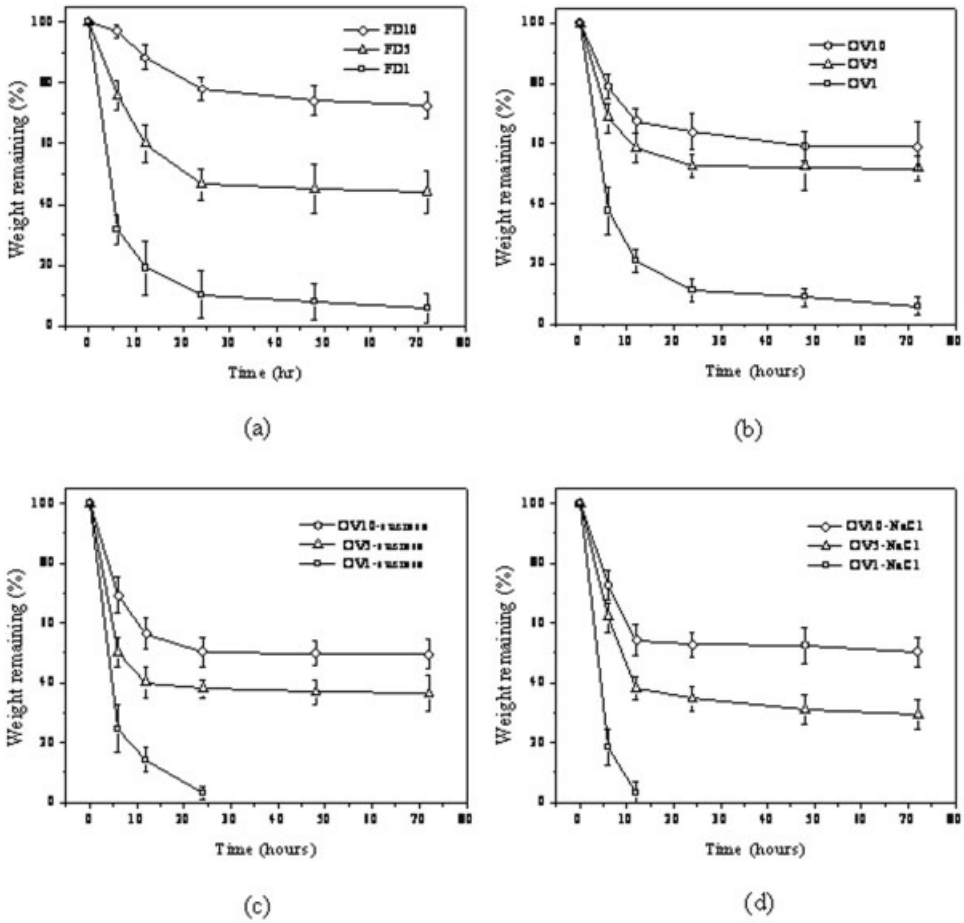

Figure 7. *In vitro* enzymatic degradation of porous gelatin scaffolds according to the matrix preparation process: (a) freeze-drying method, (b) overrun process, (c) overrun/particle (NaCl) leaching process, and (d) overrun/particle (sucrose) leaching process.

similar mechanical strength with 10 wt % freeze-dried scaffolds (FD10) [Figure 6(a)] ($p < 0.05$). This is due to the fact that overrun-processed scaffolds have a uniformly distributed dual pore structure without irregular pores formed by agglomeration of ice crystals. In the case of 5 wt % gelatin scaffolds, the mechanical strength of overrun-processed scaffolds is even greater than that of freeze-dried samples [Figure 6(b)]. In addition, except for 1 wt % gelatin scaffolds with highly porous structure (porosity, about 80–85%), combining the particle-leaching method using NaCl or sucrose caused a decrease in scaffold mechanical strength due to increased porosity ($p < 0.05$).

Enzymatic Degradation Study

Figure 7 shows the *in vitro* enzymatic degradation kinetics of gelatin scaffolds depending on the concentration of gelatin solution and preparation method. The percentage of weight loss by the gelatin scaffolds as a function of exposure time to the collagenase solution is presented. Degradation rate was significantly influenced by the concentration of gelatin solution for all samples tested. As the gelatin concentration decreased from 10 to 1 wt %, the degradation rate gradually increased.

In addition, the scaffolds prepared by the overrun/particle-leaching process were degraded faster than those prepared by the freeze-drying method and overrun process. Overrun/particle leaching-processed scaffolds prepared with 1 wt % gelatin solution were completely degraded within 24 h [Figure 7(c,d)]. The faster degradation of the overrun/particle leaching processed scaffolds was probably due to the high matrix porosity and subsequent increase in the accessibility of active sites by the enzyme, whereas the relatively slow degradation rate of freeze-dried and overrun-processed scaffolds prepared with high gelatin content might be due to the dense surface and closed pores, which can retard enzyme migration.

In vitro Cell Culture

The ability of the porous gelatin scaffolds to allow cell growth was investigated in an *in vitro* study. To access the effect of matrix structure on cellular ingrowth, human fibroblasts were cultured in gelatin scaffolds, which were prepared by different methods such as freeze-drying and overrun/particle leaching. Figure 8 shows H&E or Masson's-trichrome stained cross-sections of human fibroblasts cultured in gelatin scaffolds.

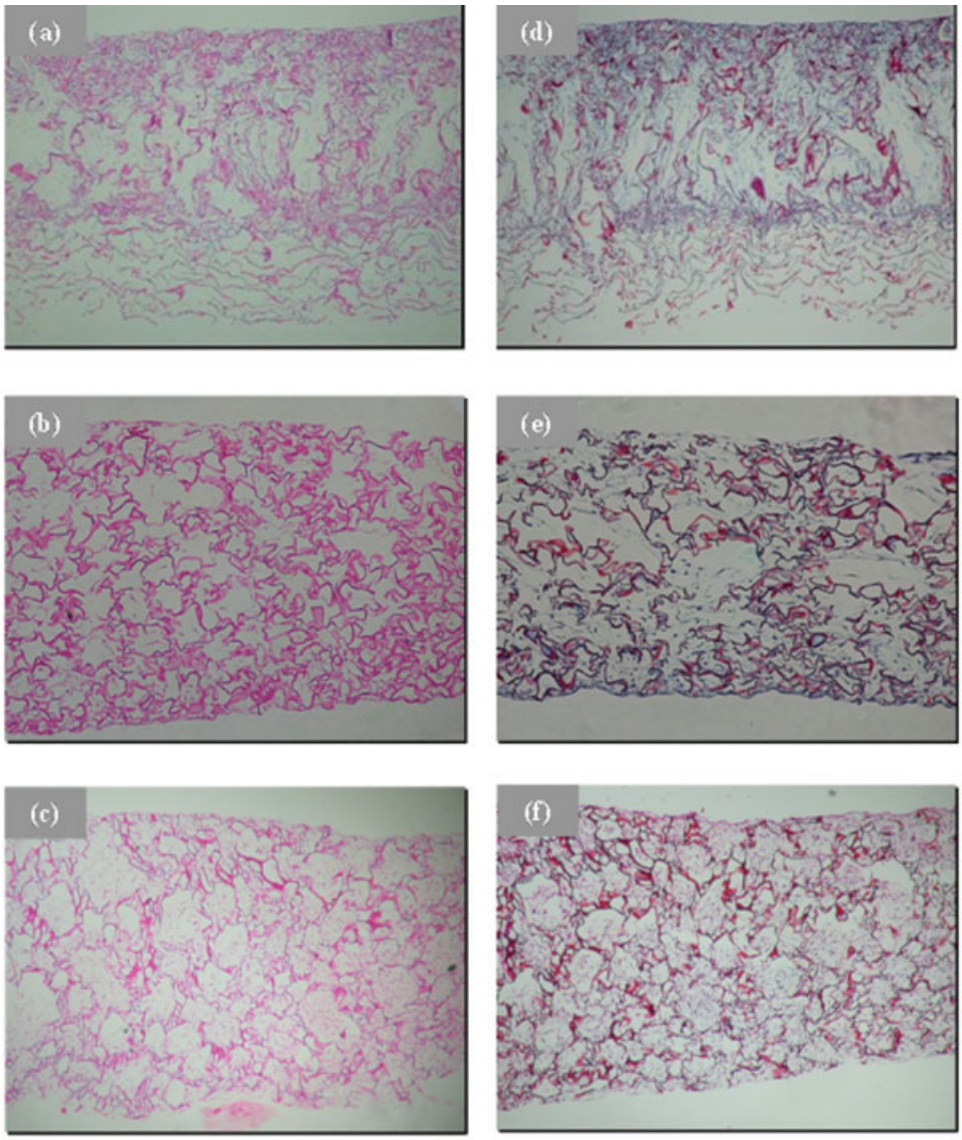

Figure 8. Histological evaluation of human fibroblasts cultured on a gelatin scaffold by H&E staining and Masson's trichrome staining: (a) H&E-stained FD5, (b) H&E-stained OV5-NaCl, (c) H&E-stained OV5-sucrose, (d) Masson's trichrome-stained FD5, (e) Masson's trichrome-stained OV5-NaCl, and (f) Masson's trichrome-stained OV5-sucrose (magnification = ×40). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

After 1 week, the fibroblasts in overrun-processed gelatin scaffolds were widely distributed on the surface of macro pores in the scaffold [Figure 8(b,c) and 8(e,f)], whereas cells seeded on freeze-dried gelatin scaffolds were mainly placed and aggregated on the top and bottom of the scaffold [Figure 8(a,d)]. Freeze-dried gelatin scaffolds might retard the migration of fibroblasts into scaffolds because of the dense surface structure. Additionally, poor interconnectivity between macropores lowered seeded cell viability and resulted in a nonuniform distribution of seeded cells.

In summary, overrun/particle leaching-processed scaffolds showed homogeneous morphology and interconnected pore structure by injected gas bubbles and added particles. In addition, they had a good affinity for fibroblasts and well-distributed cell growth throughout the three-dimensional matrix. These results indicate that the homogeneously distributed and interconnected macropores could provide enough space for cell growth within the gelatin scaffolds.

CONCLUSIONS

Porous scaffolds were designed and prepared by a novel overrun process, which is usually used in the preparation of soft ice cream. Morphological investigation revealed that overrun-processed scaffolds had closed pore structures. The closed pore structure was reformed into interconnected open pore structures by the addition of NaCl or sucrose particles.

Thus, porous gelatin scaffolds with open and interconnected pore structure can be fabricated by a new method of overrun/particle leaching. Moreover, a dense surface skin layer often observed in conventional freeze-drying method was not found on either side of the scaffold surface. Overrun-processed gelatin scaffolds showed similar mechanical strength to freeze-dried samples, although they had higher porosity. From the *in vitro* enzymatic degradation study, the degradation rate of gelatin scaffolds was influenced by the gelatin content of the initial feed solution. As the concentration of gelatin solution increased, the degradation rate gradually decreased. Fibroblasts seeded in freeze-dried scaffolds were mainly distributed on the top and bottom of the matrix due to the dense skin layer, whereas cells cultured in overrun/particle leaching-processed scaffolds distributed on the surface of macropores and homogeneously proliferated throughout the matrix. This suggests that this novel overrun/particle leaching method provides ideal three-dimensional tissue engineering matrices with a macro porous skeletal structure as well as interconnectivity between pores.

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