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Preparation and Characterization of Small Intestine Submucosa Powder Impregnated Poly(L-lactide) Scaffolds: The Application for Tissue Engineered Bone and Cartilage

Gilson Khang* and John M. Rhee

Department of Polymer Science and Technology, Chonbuk National University, 664-14, Dukjin Dong 1 Ga, Dukjin Ku, Chonju 561-756, Korea

Philkyung Shin, In Young Kim, and Bong Lee

Department of Polymer Engineering, Bukyung National University, San 100, Yongdang Dong, Nam Ku, Busan 608-023, Korea

Sang Jin Lee and Young Moo Lee

Department of Industrial Chemistry, Hanyang University, 17 Haengdang Dong, Seongdong Ku, Seoul 133-791, Korea

Hai Bang Lee

Biomaterials Laboratory, Korea Research Institutes of Chemical Technology, P.O. Box 107, Yusung, Daejon 305-606, Korea

Ilwoo Lee

Department of Neurosurgery, Catholic University Medical College, 520-2 Deaheung Dong, Jung Ku, Daejon 301-723, Korea

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Abstract: In order to endow with new bioactive functionality from small intestine submucosa (SIS) powder as natural source to poly(L-lactide) (PLA) and poly(lactide-*co*-glycolide) (PLGA) synthetic biodegradable polymer, porous SIS/PLA and SIS/PLGA as natural/synthetic composite scaffolds were prepared by means of the solvent casting/salt leaching methods for the possibility of the application of tissue engineered bone and cartilage. A uniform distribution of good interconnected pores from the surface to core region was observed the pore size of 40~500 μm independent with SIS amount using the solvent casting/salt leaching method. Porosities, specific pore areas as well as pore size distribution also were almost same. After the fabrication of SIS/PLA hybrid scaffolds, the wetting properties was greatly enhanced resulting in more uniform cell seeding and distribution. Five groups as PGA non-woven mesh without glutaraldehyde (GA) treatment, PLA scaffold without or with GA treatment, and SIS/PLA (Code No. 3 ; 1 : 12 of salt content, 0.4 : 1 of SIS content, and 144 μm of median pore size) without or with GA treatment were implanted into the back of nude mouse to observe the effect of SIS on the induction of cells proliferation by hematoxylin and eosin, and von Kossa staining for 8 weeks. It was observed that the effect of SIS/PLA scaffolds with GA treatment on bone induction are stronger than PLA scaffolds, that is to say, in the order of PLA/SIS scaffolds with GA treatment > PLA/SIS scaffolds without GA treatment > PGA nonwoven > PLA scaffolds only with GA treatment = PLA scaffolds only without GA treatment for the osteoinduction activity. The possible explanations are (1) many kinds of secreted, circulating, and extracellular matrix-bound growth factors from SIS to significantly affect critical processes of tissue development and differentiation, (2) the exposure of SIS to GA resulted in significantly calcification, and (3) peri-implant fibrosis due to covalent bonding between collagen molecule by crosslinking reaction. In conclusion, it seems that SIS plays an important role for bone induction in SIS/PLA scaffolds for the application of tissue engineering area.

Keywords : SIS, PLA, tissue engineering, bone, scaffolds.

*e-mail : gskhang@moak.chonbuk.ac.kr

Introduction

It has been recognized that tissue engineering offers an alternative techniques to whole organ and tissue transplantation for diseased, failed or malfunctioned organs. To reconstruct a new tissues, cells which is harvested and dissociated from the donor tissue including nerve, liver, pancreas, cartilage and bone, and scaffold substrates which cells are attached and cultured resulting in the implantation at the desired site of the functioning tissue must be needed. Recently, the family of poly(α-hydroxy acid)s such as polyglycolide (PGA), polylactide (PLA) and its copolymer like poly(lactide-*co*-glycolide) (PLGA) which are only the synthetic polymers approved for human clinical use by FDA are extensively used or tested for the scaffolds materials as a bioerodible material due to good biocompatibility, controllable biodegradabilitiy, and relatively good processability.[1] These polymers degrade by nonspecific hydrolytic scission of their ester bonds.[2] The hydrolysis of PLA yields lactic acid which is a normal byproduct of anaerobic metabolism in human body and is corporated in the tricarboxylic acid (TCA) cycle to be finally excreted by the body as carbon dioxide and water. PGA biodegrades by a combination of hydrolytic scission and enzymatic (esterase) action producing glycolic acid[3] which can either enter the TCA cycle or be excreted in urine and be eliminated as carbon dioxide and water.[4] The degradation time of PLGA can be controlled from weeks to over a year by varying the ratio of monomers and the processing conditions.[5] It might be a suitable biomaterial for use in tissue engineered repair systems[6-12] in which cells are implanted within PLGA films or scaffolds and in drug delivery systems[13-16] in which drugs are loaded within PLGA microspheres.

However, it is more desirable to endow with new functionality for the PLA, PGA and PLGA scaffold for the applications of cell and tissue engineering.[17-24] For example, hydrophobic surfaces of PLA, PGA, and PLGA possess high interfacial free energy in aqueous solutions, which tend to unfavorably influence their cell-, tissue- and blood-compatibility in initial stage of contact, so, it might be more favorable to hydrophilic surface resulting in more uniform cell seeding and distribution. Another example, the bioactive materials impregnated scaffolds might be better for the cell proliferation, differentiation, and migration due to the stimulation of cell growth from the sustained release of cytokine molecules such as nerve growth factor and vascular endothelial cell growth factor (VEGF).[25]

One of the significant natural bioactive materials is small intestine submucosa (SIS) powder.[26-34] Badylak *et al.*[26-28] described systematically that an acellular resorbable scaffold material derived from the SIS has been shown to be rapidly resorbed, support early and abundant new blood vessel growth, and serve as a template for the constructive remodeling of several body tissue including musculoskeletal structures, skin, body wall, dura mater, urinary bladder and blood vessels. The SIS material consists of naturally occurring extracellular matrix (ECM) that has been shown to be rich in components which support angiogenesis such as fibronectin, glycosaminoglycans including heparin, several collagens including Types I, III, IV, V and VI, and angiogenic growth factors such as basic fibroblast growth factor and VEGF.[26] Microporous biodegradable polymeric scaffolds impregnated with bioactive materials have been prepared by several techniques including solvent casting/salt leaching,[35] phase separation,[36] solvent evaporation,[37] and emulsion freeze drying method[21,23,37] in order to maximize cell seeding, attachment, growth, extracellular matrix production, vascularization and tissue ingrowth.

In this study, we developed the novel natural/synthetic composite scaffold like SIS impregnated PLA and PLGA (SIS/PLA and SIS/PLGA, respectively) scaffolds for the possibility of the application of the tissue engineered bone and cartilage. SIS/PLA and SIS/PLGA scaffolds were prepared by solvent casting/salt leaching method. Scaffolds were characterized by scanning electron microscopy (SEM) and mercury intrusion porosimeter. Also the effect of SIS on bone formation from the SIS/PLA scaffolds was observed by the implantation onto the back of the athymic nude mouse.

Experimental

Materials. PLA (molecular weight : 110,000 g/mole, Resomer® L206) and PLGA (molecular weight : 90,000 g/mole, 75 : 25 by mole ratio of lactide to glycolide, Resomer® RG756) were purchased from Boehringer Ingelheim (Ingelheim, Germany).

PGA nonwoven (density : 50 mg/cc and thickness : 3.0 mm) was purchased from Albany International Co. Ltd. (USA). Ammonium bicarbonate (Oriental Chem. Co., Korea) as a porogen and methylene chloride (MC, Tedia Co. Inc., USA) as an organic solvent for PLA and PLGA were used as received. All other chemicals were a reagent grade.

Preparation of SIS. Sections of porcine jejunum were harvested from market pigs within 10 minutes of sacrifice and prepared according to the method of Badylak *et al.* Briefly, the harvested sections of intestine were rinsed free of contents and split in the longitudinal direction to form an elongated sheet. The superficial layers of the tunica mucosa were then removed by mechanical delamination using back of knife. The tissue was then turned to the opposite side and the tunica muscularis externa and tunica serosa layers were mechanically removed again. The remaining tissue represented the SIS and consisted of the tunica submucosa and basilar layers of the tunica mucosa. The material consisted almost entirely of extracellular matrix. Following lysis of the few cellular remnants by exposure to 0.1% peracetic acid, the SIS materials was rinsed extensively in phosphate buffered saline (PBS, Sigma Chem. Co., USA; pH = 7.4)

and deionized water. The remaining SIS material was approximately 80 ~ 150 μm thickness as shown in Figure 1. This acellular material consisted of the natural components of the ECM left in the normal three-dimensional architecture and then dried to evaporate water at 70 °C for 24 hrs using freeze dryer (Model FDU-540, EYELA, Japan). Samples were stored in a vacuum dessicator at room temperature for at least 7 days. These dried SISs were pulverized using freezer mill (SPEX 6700, Mutchen, USA) at -198 °C to get 10 ~ 20 μm size of SIS powder for the improvement of dispersivity into PLA and PLGA matrix.

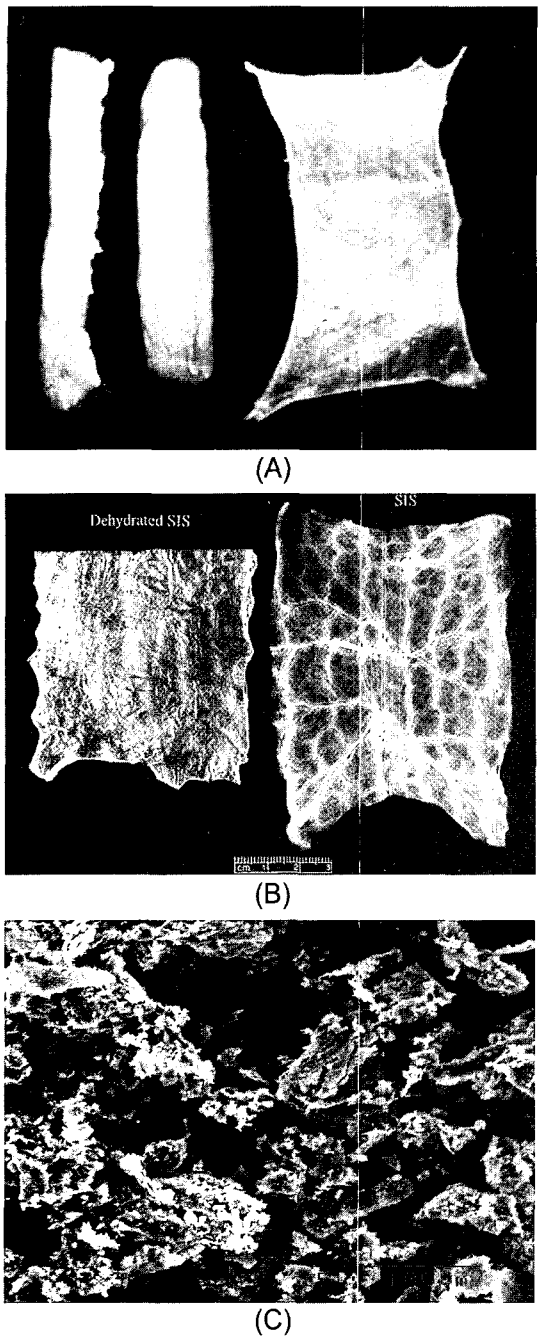


Figure 1. Schematic diagram of the preparation processing of SIS. (A) Section of porcine jejunum, (B) SIS, and (C) SEM microphotographs after freezer-milled SIS (original magnification ; × 100).

Preparation of Scaffold by Solvent Casting/Salt Leaching Method. Scaffold was prepared by solvent casting/salt leaching methods from mixtures composed of PLA and PLGA as biodegradable matrix, ammonium bicarbonate as porogen, and SIS as natural matrix containing bioactive molecule as shown the schematic diagram in Figure 2. First, 10, 12 and 14 grams of ammonium bicarbonate particles sieved to a size range of 180 to 250 μm and 250 to 425 μm to 1 gram of PLA were added to a solution of 20 w/v% concentration of PLA in MC. SIS with 0.3, 0.4 and 0.5 grams was thoroughly dispersed into this PLA solution (to 1 gram PLA base) and then dispersions were cast in a silicon mold like circular disk (diameter : 5 mm and thickness : 3 mm). Processing variables for the fabrication of SIS/PLA scaffolds were listed in Table I. The samples were air-dried for 48 hrs and subsequently vacuum-dried for 24 hrs to remove any remained solvent. The resulting SIS impregnated PLA scaffolds were immersed in deionized water for 48 hrs with changed every 6 hrs to leach out ammonium bicarbonate, then finally vacuum-dried. These totally dried SIS/PLA scaffolds were stored in a dessicator under vacuum until use. Also, SIS/PLGA scaffolds were prepared for the same porcesses with SIS/PLA scaffolds.

SEM Observations. Surface and cross sectional morphology of SIS/PLA and SIS/PLGA scaffolds were observed by SEM (Model S-2250N, Hitachi Co. Ltd., Japan) to investigate the pore structure and pore size. Samples sliced by sharp razor for SEM were mounted on metal stub with double-sided tape and coated with platinum for 30 second under argon atmosphere using plasma sputter (SC 500K,

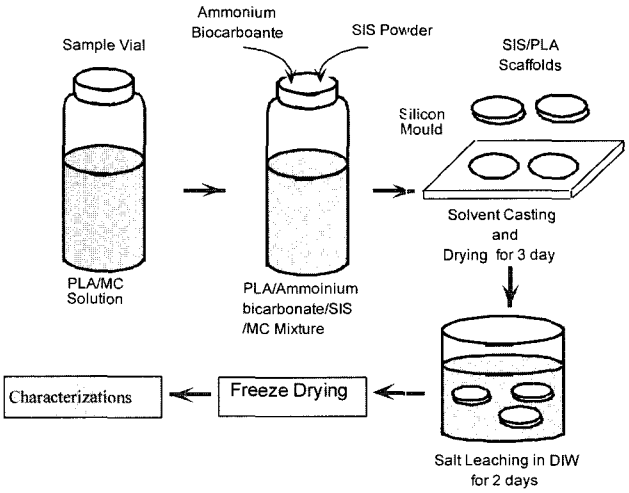


Figure 2. Schematic diagram of the solvent casting/salt leaching method to fabricate SIS/PLA scaffolds.

Table I. Processing Variables and Properties of SIS/PLA and SIS/PLGA Scaffolds by means of Solvent Casting/Salt Leaching Method

Code	Mixture Ratios			Salt Size (μm)	Median Pore Diameter (μm)	Porosity (%)
	Polymers	Salt Content (g)	SIS Content (g)			
L1	PLA	10	0.4	250-425	175.8	88.1
L2	PLA	12	0.4	180-250	97.8	93.4
L3	PLA	12	0.4	250-425	144.4	92.0
L4	PLA	14	0.4	250-425	156.1	92.6
L5	PLA	12	0.3	250-425	204.6	84.4
L6	PLA	12	0.4	250-425	170.5	89.5
L7	PLA	12	0.5	250-425	140.3	86.6
G1	PLGA	12	-	250-425	93.3	93.2
G2	PLGA	12	0.4	250-425	136.5	83.8

Emscope, UK). The size and size distribution of pore were determined according to a reference scale.

Mercury Intrusion Porosimeter Analysis. SIS/PLA and SIS/PLGA scaffolds were analyzed by mercury intrusion porosimeter using an AutoPore II 9220 (Micromeritics Co. Ltd., USA) to determine pore size distributions, specific pore area, median pore diameter and porosity. A solid penometer volume was ranged with 6.7 ~ 7.3 mL and 0.1 g of sample was analyzed. Mercury was filled from a filling pressure of 3.4 kPa and intruded to a maximum pressure of 414 MPa. The relationship between the filling pressure and pore radius is given by the Washburn equation [38];

$$R = -2\gamma\cos\theta/P \quad (1)$$

where γ is the surface tension of mercury (~ 460 dyne/cm) and θ is the contact angle between mercury and polymer surface. For PLA and PLGA, the value of mercury contact angle was measured at 25 °C as 160 degree using contact angle goniometer (Model 100-0, Rame-Hart Inc., USA). The porosity, ε, was calculated from the total intrusion volume (per unit mass), V_i, and the skeletal density, ρ, as [39];

$$\varepsilon = V_i/(V_i + 1/\rho) \quad (2)$$

Measurement of Wetting Property of SIS Impregnated Scaffolds. In order to compare the wetting property of control and SIS/PLA and SIS/PLGA scaffolds, 0.05 w/v% blue dye solution was dropped onto scaffolds and then photographs were taken.

Implantation of SIS/PLA Scaffolds. Five groups as PGA nonwoven mesh without glutaraldehyde (GA, Sigma Chem. Co.) treatment, PLA scaffold without or with GA treatment, and SIS/PLA (Code No. 3; 1 : 12 of salt content, 0.4 : 1 of SIS content, and 144 μm of median pore size) without or with GA treatment were implanted into the back of nude mouse to observe the effect of SIS on the induction of chondrogenesis and osteogenesis compared with control PLA scaffolds. SIS/PLA scaffolds were treated with 0.25% GA for 20 min, then rinsed three times for 2 hrs with sterile water and then freeze-dried at 70 °C for 24 hrs. The implants sterilized with ethylene oxide gas were removed after 8 weeks and fixed in 10% buffered formalin. Thin sections were cut from paraffin embedded tissue and histological sections were stained hematoxylin and eosin (H & E) and von Kossa staining. Photomicrographs were taken using a Nikon inverted microscope with 100 and 400 magnifications.

Results and Discussion

In order to endow with new bioactive functionality from SIS as natural source to PLA and PLGA synthetic biodegradable polymer, porous SIS/PLA and SIS/PLGA as natural /synthetic composite scaffolds were prepared by means of the solvent casting/salt leaching methods for the possibility of the application of tissue engineered bone and cartilage. It has been recognized that SIS contains naturally occurring many kinds of secreted, circulating, and extracellular matrix-bound growth factors as platelet derived growth factor, epidermal growth factor, transforming growth factor, basic fibroblast growth factor and VEGF, and extracellular matrix as fibronectin, glycosaminoglycans including heparin, as well as several collagens including Types I, III, IV, V and VI.[26] Also, SIS was widely used and tested for skin substitutes, a filling matrix for cartilage defects in clinic due to the improvement availability through the commercialization by DePuy and Cook. Figure 1(C) shows the pulverized SIS using freezer mill that the size of 10 ~ 20 μm for the enhancement of dispersivity into PLA matrix.

Effect of Different Salt Contents on the Pore Structure. SEM microphotographs of SIS/PLA scaffolds with 1 : 10, 1 : 12 and 1 : 14 of the ratio of polymer to SIS by means of solvent casting/salt leaching method are shown in Figure 3. SIS (0.4 g) and PLA or PLGA (1 g) to ammonium bicar-

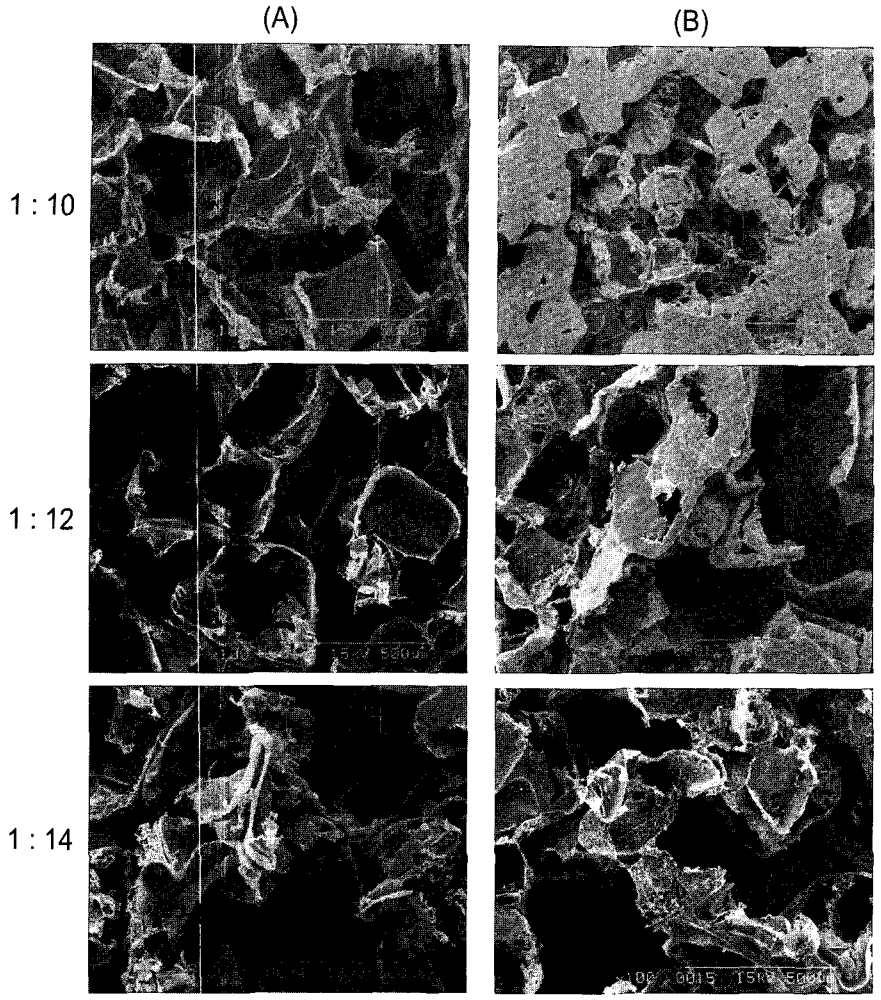


Figure 3. SEM microphotographs of (A) cross section and (B) surface for SIS/PLA and SIS/PLGA scaffolds with different salt contents. (1 : 10, 1 : 12 and 1 : 14 by weight ratio of polymer to salt, respectively) (original magnifications × 100).

bonate were fixed and then ammonium bicarbonate contents were varied with 10 g (L1), 12 g (L3) and 14 g (L4). Figures 4 and 5 show the effects of the different salt size (180 ~ 250 μm and 250 ~ 425 μm of ammonium bicarbonate sizes) and the different SIS content (1 : 0.3, 1 : 0.4 and 1 : 0.5 by weight ratio of polymer to SIS) on the pore morphology of cross section and surface for SIS/PLA scaffolds, respectively. All of surface, cross section, and side of SIS/PLA scaffolds were highly porous with good interconnections between pores in which can support the surface of cell growth, proliferation and differentiation. Particularly, it was observed a uniform distribution of well interconnected pores from the surface to core region due to the characteristics of salt leaching method. It can be observed that the pore size was almost same like 175.8 μm for L1, 144.4 μm for L3, and 156.1 μm for L4 as listed in Table I. Figure 6 shows the effect of different salt content on pore size distribution of PLA/SIS scaffolds analyzed by mercury porosity meter. It was observed almost same pore size distribution and in the range of 10 μm as the

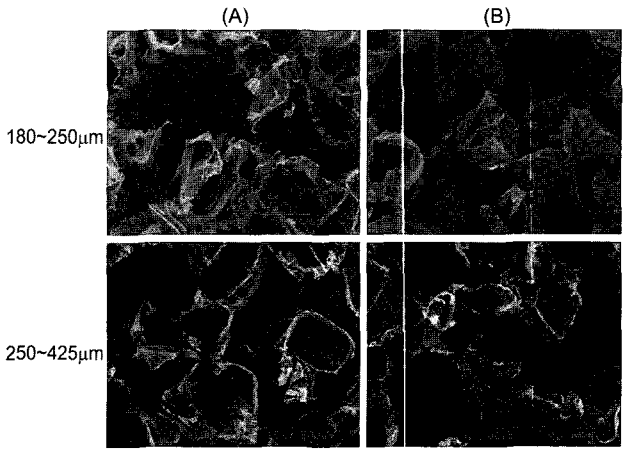


Figure 4. SEM microphotographs of (A) cross section and (B) surface for SIS/PLA and SIS/PLGA scaffolds with different salt size. (180~250 μm and 250 ~ 425 μm of ammonium bicarbonate sizes, respectively) (original magnifications × 100).

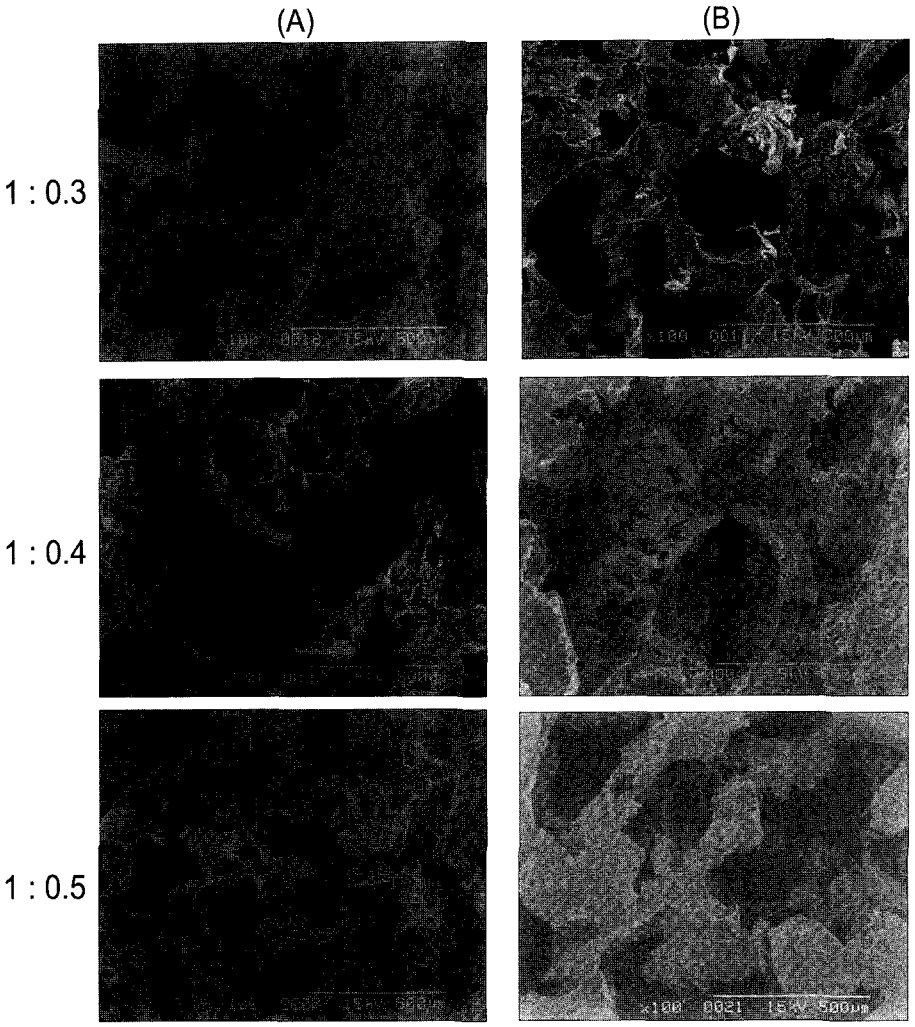


Figure 5. SEM microphotographs of (A) cross section and (B) surface for SIS/PLA and SIS/PLGA scaffolds with different SIS contents. (1 : 0.3, 1 : 0.4 and 1 : 0.5 by weight ratio of polymer to SIS, respectively) (original magnifications × 100).

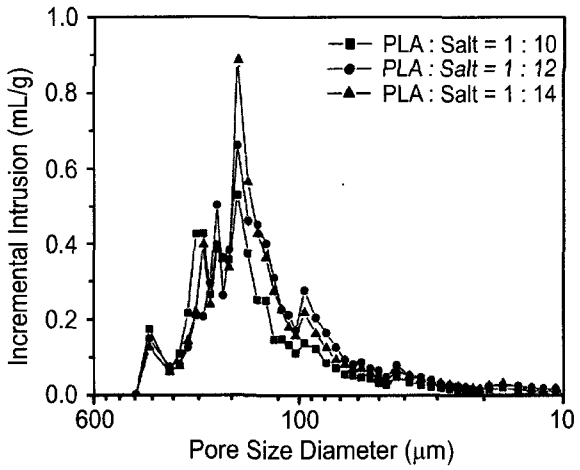


Figure 6. Effect of different salt content on pore size distribution of SIS/PLA scaffolds by means of the solvent casting/salt leaching method.

smallest to 450 μm as the largest pore size. Porosities also were almost same around 88 ~ 92%, that is to say, the variation of the salt amount from 10 g to 14 g on the basis of 1 g of PLA did not affect the pore size and pore size distribution. Figures 7 to 9 show the effect of different manufacturing condition on pore size distribution of SIS/PLA and SIS/PLGA scaffolds. It observed that the size distribution of SIS/PLA and SIS/PLGA scaffolds was constantly uniform since the size distribution of a porogen as ammonium bicarbonate was also same resulting in relatively large surface area per volume, higher porosity, almost same interconnective structure between pore, more uniform the pore size and pore size distribution, no change pore size and distribution varied with SIS contents, and less processing variables compared with other preparing methods.

Figure 10 shows the wetting property of SIS impregnated PLGA and PLA scaffolds. As shown in figure, the drop of blue dye solution was easily wetted in SIS/PLGA and PLA

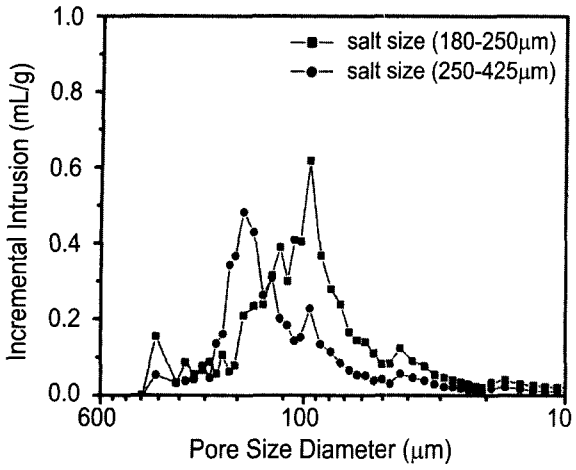


Figure 7. Effect of different salt size on pore size distribution of SIS/PLA scaffolds by means of the solvent casting/salt leaching method.

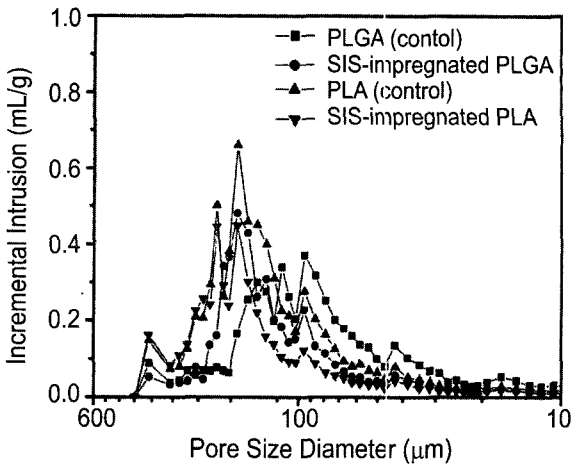


Figure 9. Comparison of pore size distribution between PLGA and SIS/PLGA, and PLA and SIS/PLA scaffolds.

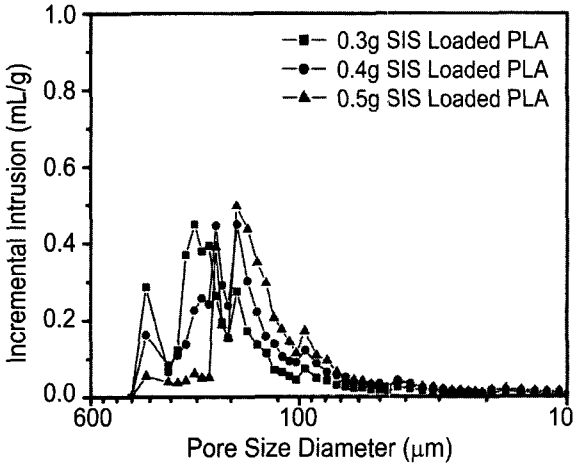


Figure 8. Effect of different SIS content on pore size distribution of SIS/PLA scaffolds by means of the solvent casting/salt leaching method.

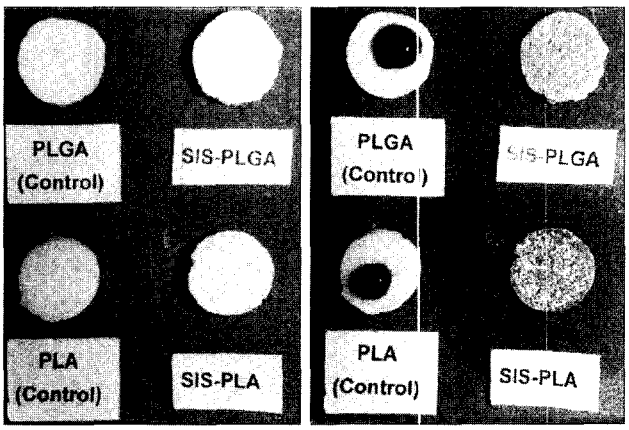


Figure 10. The wetting experiment by the drop of blue dye solution.

scaffolds within second due to more hydrophillized surface by collagen SIS powder. From these results, it might be expected more fast penetration of cell culture medium, better uniform cell seeding and distribution, and better cell migration and growth.[17-25]

The physical and chemical requirements of ideal scaffolds for cell/tissue ingrowth are[1] (i) biocompatibility, (ii) promotion of cell adhesion, (iii) enhancement of cell growth, (iv) retention of differentiated cell function, (v) large surface area per volume, (vi) highly porosity to provide adequate space for cell seeding, growth and extracellular matrix production, and (vii) a uniformly distributed and interconnected pore structure (this factor is very important so that cells are easily distributed through the scaffolds and an organized network of tissue constituents can be formed).

Implantation of SIS/PLA Scaffolds. The scaffolds fabricated by the solvent casting/salt leaching methods were utilized to transplant and applied as a template guiding the formation of a bone and cartilage structures from the induction of osteogenesis and chondrogenesis by SIS. Five groups as PGA nonwoven mesh without GA treatment (Figure 11(A)), PLA scaffold without (Figure 11(B)) or with GA treatment (Figure 11(C)), and 0.5 : 1 SIS/PLA without (Figure 11(D)) or with GA treatment (Figures 11(E) and (F)), were implanted on the back of athymic nude mouse to investigate the effect of SIS on the induction of cells proliferation for 8 weeks as shown in Figure 11 for the each microphotograph of von Kossa staining sections. In PGA scaffold (Figure 11(A)), the calcified bone stained by von Kossa was observed around PGA scaffolds, however, there was no evidence of new bone formation inner part of PGA scaffolds. It might be suggested that the hydrophobicity of PGA nonwoven could not permit to migrate the undifferentiated stem cells at the subcutaneous

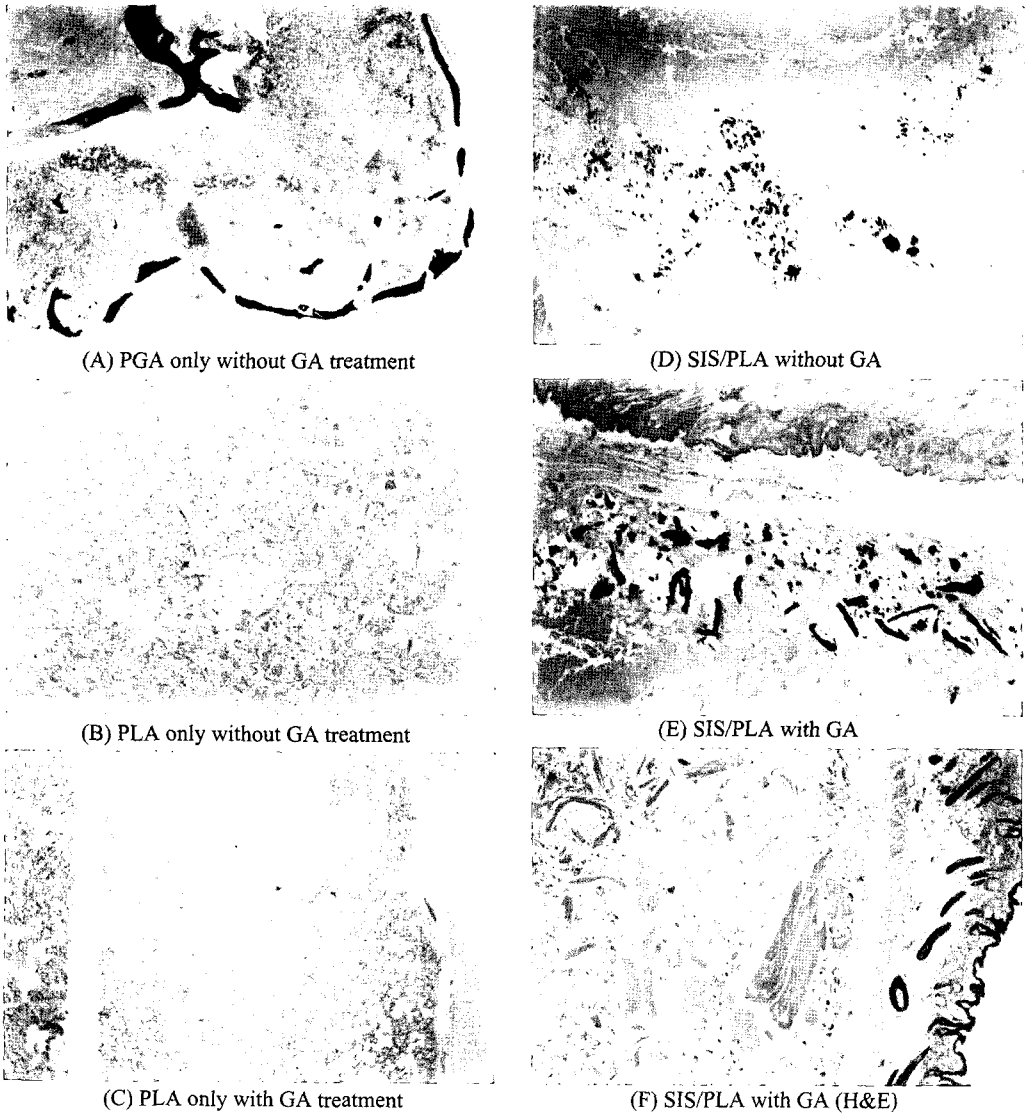
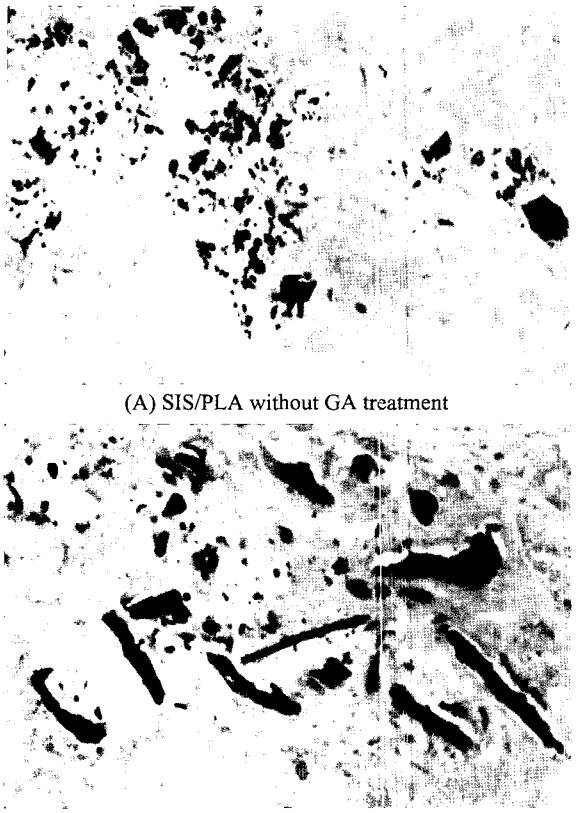


Figure 11. Photomicrographs of von Kossa and H&E histological sections of implanted (A) PGA nonwoven, (B) PLA scaffold only without GA treatment, (C) PLA scaffold only with GA treatment, (D) SIS/PLA scaffold without GA treatment, (E) SIS/PLA scaffold with GA treatment, and (F) SIS/PLA scaffold with GA treatment (H&E). (original magnifications × 100).

sites into PGA scaffold resulting in the bone formation of the vicinity of scaffolds. In addition, it was observed that there was no evidence of new bone formation at PLA scaffold without or with GA treatment as shown in Figures 11(B) and (C) due to no biological activity and functionality of PLA. However, we can observe the evidence of calcification by the bioactivity of SIS/PLA and SIS/PLGA scaffolds from the undifferentiated stem cells in the subcutaneous sites and other soft connective tissue sites[29] having a preponderance of stem cells compared with PLA only and PGA only as shown in Figures 11(D) and (E) for von Kossa staining and Figure 11(F) for H & E staining. (Data of SIS/PLGA were not shown.) Badylak *et al.*[26-29] have successfully carried out the isolation and the identification of many kinds of secreted, circulating, and extracellular matrix-bound growth factors from SIS. So, it might be explained that these growth factors are significantly affected the critical processes of tissue development and differentiation, that is to say, the osteogenesis and chondrogenesis of the undifferentiated stem cells in the subcutaneous sites and other soft connective tissue sites having a preponderance of stem cells.[30-33]

In order to observe the difference between with and without GA treatment, the higher magnified photos (× 400) of Figures 11(D) and (E) are shown in Figures 12(A) and (B), respectively. We can observe the more complete bone formation with GA treated SIS/PLA hybrid scaffold compared with no GA treatment. Possible explanation is the exposure of SIS to GA resulted in significant calcification as well as



(A) SIS/PLA without GA treatment

(B) SIS/PLA with GA treatment

Figure 12. Photomicrographs of von Kossa and H&E histological sections of implanted (A) SIS/PLA scaffold without GA treatment and (B) SIS/PLA scaffold with GA treatment. (original magnifications × 400).

peri-implant fibrosis due to covalent bonding between collagen molecule by crosslinking reaction.[29] It has been recognized that these peri-implant fibrosis can be altered the healing characteristics of SIS. Advantages of GA treatment for SIS are the reduction of the potential xenogenic and allogenic biomaterials and the improvement of the preserving strength of the devices as well as the controlling resorption time.[30] In summary, it seems that SIS plays an important role for bone and cartilage induction in SIS/PLA scaffolds rather than only synthetic polymer.

Studies on the more detailed mechanism of osteoinduction and chondroinduction of SIS/PLA scaffolds, the quantification of osteoinduction such as calcium contents and alkaline phosphatase activity, the biodegradation test, the implantation of SIS/PLA scaffolds with mesenchymal stem cell, the optimal ratio of SIS to polymer, the optimal pore size and size distribution of SIS/PLA scaffolds for the application of tissue engineered bone and cartilage, and its relative animal experiment are in progress.

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