Korea Polymer Journal, Vol. 9, No. 5, pp 267 – 276 (2001)

Preparation and Characterization of Demineralized Bone Particle Impregnated Poly(L-lactide) Scaffolds

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Received July 23, 2001

Abstract : In order to endow with new bioactive functionality from demineralized bone particle (DBP) as natural source to poly(L-lactide) (PLA) synthetic biodegradable polymer, porous DBP/PLA as natural/synthetic composite scaffolds were prepared and compared by means of the emulsion freeze drying and solvent casting/salt leaching methods for the possibility of the application of tissue engineered bone and cartilage. For the emulsion freeze drying method, it was observed that the pore size decreased in the order of 79μm (PLA control) > 47 μm (20% of DBP) > 23 μm (40% of DBP) > 15 μm (80% of DBP). Porosities as well as specific pore areas decreased with increasing the amount of DBP. It can be explained that DBP acts like emulsifier resulting in stabilizing water droplet in emulsion. For the solvent casting/salt leaching method, a uniform distribution of well interconnected pores from the surface to core region were observed the pore size of 80~70 μm independent with DBP amount. Porosities as well as specific pore areas also were almost same. For pore size distribution by the mercury intrusion porosimeter analysis between the two methods, the pore size distribution of the emulsion freeze drying method was broader than that of the solvent casting/salt leaching method due to the mechanism of emulsion formation. Scaffolds of PLA alone, DBP/PLA of 40 and 80%, and DBP powder were implanted on the back of athymic nude mouse to observe the effect of DBP on the induction of cells proliferation by hematoxylin and eosin staining for 8 weeks. It was observed that the effect of DBP/PLA scaffolds on bone induction are stronger than PLA scaffolds, even though the bone induction effect of DBP/PLA scaffold might be lowered than only DBP powder, that is to say, in the order of DBP only > DBP/PLA scaffolds of 40 and 80% DBP > PLA scaffolds only for osteoinduction activity. In conclusion, it seems that DBP plays an important role for bone induction in DBP/PLA scaffolds for the application of tissue engineering area.

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 new tissues, cells which are 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-

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hydroxy acid)s such as polyglycolide (PGA), polylactide (PLA) and its copolymer like poly(lactide-*co*-glycolide) (PLGA) which are among the few 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 make surface hydrophilic, resulting in more uniform cell seedng 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 growing from the sustained release of cytokine molecules such as nerve growth factor and vascular endothelial cell growth factor.[25]

One of the significant natural bioactive materials is demineralized bone particle (DBP) whose has a powerful inducer of new bone growth. Urist[26,27] described firstly the sequence of bone induction using demineralized cortical bone matrix and reported that bone morphogenetic protein acts as local mitogen to stimulate proliferation of mesenchymal stem cells.[28-34] Macroporous 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 DBP impregnated PLA (DBP/PLA) scaffolds for the possibility of the application of the tissue engineered bone and cartilage. DBP/PLA scaffolds were prepared and compared by solvent casting/salt leaching method and emulsion freeze drying method. Scaffolds were characterized by scanning electron microscopy (SEM) and mercury intrusion porosimeter. Also the effect of DBP on bone formation from the DBP/PLA scaffolds was observed by the implantation onto the athymic nude mouse.

Experimental

Materials. PLA (molecular weight : 90,000 g/mole) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Human DBP was obtained from Osteotech, Inc. (Shrewsbury, USA). DBP with 10~30 μm size from 300 μm was milled to pulverize using freezer mill (SPEX 6700, USA). Ammonium bicarbonate (Oriental Chem. Co., Korea) and methylene chloride (MC, Tedia Co. Inc., USA) were used as received. All other chemicals were a reagent grade.

Preparation of DBP/PLA Scaffolds by Emulsion Freeze Drying Method. A schematic diagram of the fabrication process by emulsion freeze drying method is shown in Figure 1. PLA 2.4 g was firstly dissolved in 24 mL of MC. Then 20, 40, and 80 wt% of DBP was dispersed in 5 mL of distilled water. These DBP contained distilled water put into PLA solution as a 60 v/v% concentration of PLA solution to water, then was thoroughly dispersed by a sonication for 15 second with 50 W power by means of sonicator. Pro-

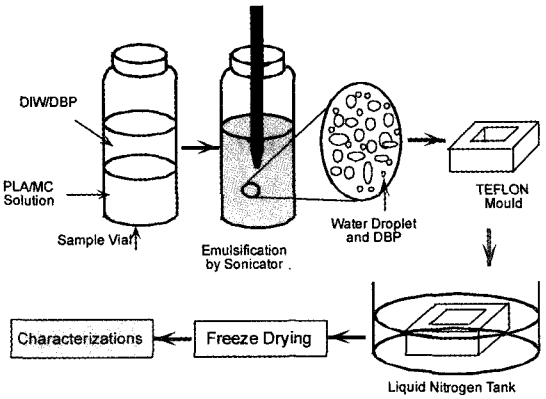

Figure 1. Schematic diagram of the emulsion freeze drying method to fabricate DBP/PLA scaffolds.

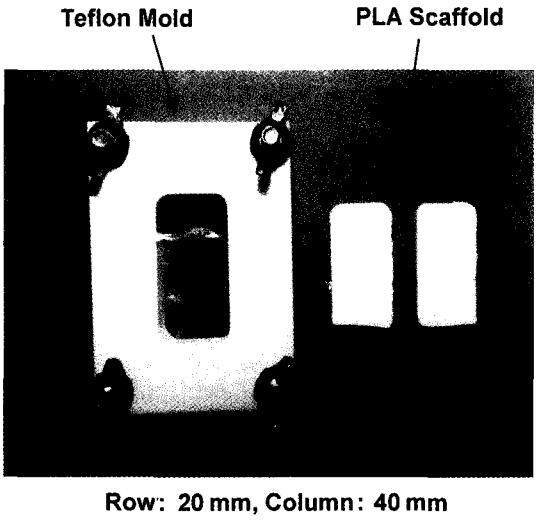

Figure 2. Teflon mold for the emulsion freeze drying method to fabricate DBP/PLA scaffolds.

cessing variables for the fabrication of DBP/PLA scaffolds were listed in Table I. These emulsions containing with DBP poured in to Teflon mould (20 mm × 40 mm × 5 mm depth, and see Figure 2) and quenched quickly into liquid nitrogen at

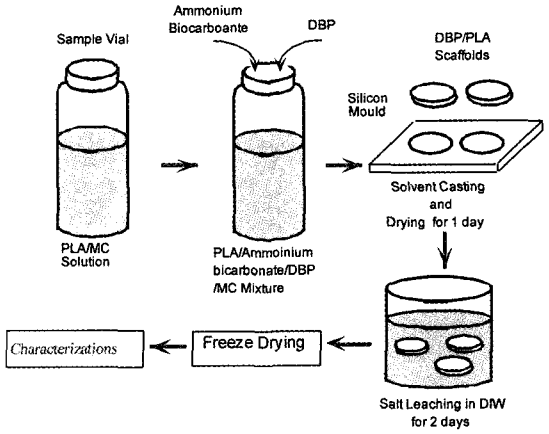

Figure 3. Schematic diagram of the solvent casting/salt leaching method to fabricate DBP/PLA scaffolds.

-198°C. The resulting DBP/PLA scaffolds were dried to evaporated MC at -70°C for 24 hrs using freeze dryer (Model FDU-540, EYELA, Japan). Samples were placed in a vacuum dessicator at room temperature for at least 7 days to remove residual solvents.

Preparation of DBP/PLA Scaffolds by Solvent Casting/Salt Leaching Method. Scaffolds were also prepared by solvent casting/salt leaching methods from mixtures composed of PLA as biodegradable matrix, ammonium bicarbonate as porogen, and DBP as natural matrix containing bioactive molecule as shown the schematic diagram in Figure 3. First, 90 w/w% of ammonium bicarbonate particles sieved to a size range of 180 to 250 μm to PLA were added to a solution of 20 w/v% concentration of PLA in MC. DBP was thoroughly dispersed into this PLA solution with 20, 40 and 80% and then dispersions were cast in a silicon mold (diameter : 5 mm and thickness : 3 mm). Processing variables for the fabrication of DBP/PLA scaffolds were listed in Table II. The samples were air-dried for 48 hrs and

Table I. Processing Variables and Properties of DBP/PLA Scaffolds by means of Emulsion Freeze Drying Method

PLA Conc. (w/v%)	DBP Content (%)	Volume of PLA Solution to Water (v/v%)	Porosity (%)	Median Pore Size (μm)	Specific Pore Area (m^2/g)
10	0	60	91.12	79	43.33
10	20	60	87.87	47	27.47
10	40	60	85.32	23	19.18
10	80	60	77.01	15	11.79

Table II. Processing Variables and Properties of DBP/PLA Scaffolds by means of Solvent Casting/Salt Leaching

PLA Conc. (w/v%)	DBP Content (%)	Volume of PLA to AB[a] (w/w%)	Porosity (%)	Median Pore Size (μm)	Specific Pore Area (m^2/g)
20	0	90	97.86	81	68.29
20	20	90	96.02	76	65.21
20	40	90	94.66	74	64.95
20	80	90	94.37	69	62.75

[a]AB : Ammonium bicarbonate.

subsequently vacuum-dried for 24 hrs to remove any remaining solvent. The resulting DBP impregnated PLA scaffolds were immersed in deionized water for 24 hrs with changed every 6 hrs to leach out ammonium bicarbonate, then finally vacuum-dried. These totally dried scaffolds were stored in a dessicator under vacuum until use.

SEM Observations. Surface and cross sectional morphology DBP/PLA scaffolds were observed by SEM (Model S-2250N, Hitachi Co. Ltd., Japan) to investigate the pore structure and pore size prepared by both emulsion freeze drying and solvent casting/salt leaching method. 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 500 K, Emscope, UK). The size and size distribution of pore were determined according to a reference scale.

Mercury Intrusion Porosimeter Analysis. DBP/PLA 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[39];

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

where γ is the surface tension of mercury (~ 460 dyne/cm) and θ is the contact angle between mercury and polymer surface. For PLA, the value of was measured at 25°C as 160° 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[40];

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

Implantation of DBP/PLA Scaffolds. Four groups like DBP/PLA scaffolds with 40 and 80% concentration of DBP, DBP, and PLA scaffold were implanted into the back of athymic nude mouse to observe the effect of DBP on the induction of osteogenesis compared with control PLA scaffolds. 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). Photomicrographs were taken using a Nikon inverted microscope with 100 magnifications.

Results and Discussion

In order to endow with new bioactive functionality from DBP as natural source to PLA synthetic biodegradable polymer, porous DBP/PLA as natural/synthetic composite scaffolds were prepared and compared by means of the emulsion freeze drying and solvent casting/salt leaching methods for the possibility of the application of tissue engineered bone and cartilage. It has been recognized that DBP contains many kinds of osteogenic and chondrogenic cytokines as bone morphogenetic protein and widely uses a filling agent for bony defects in clinic due to improved availability through the growing tissue bank industry. Figure 4 shows the pulverized DBP using freezer mill that the size decreased from 100~300 μm to 10~20 μm for the improvement of dispersivity into PLA matrix.

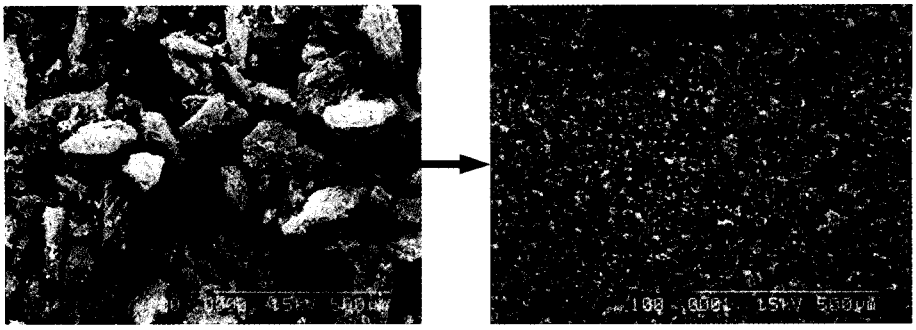

Figure 4. SEM microphotographs before and after pulverized DBP (original magnification ; ×100).

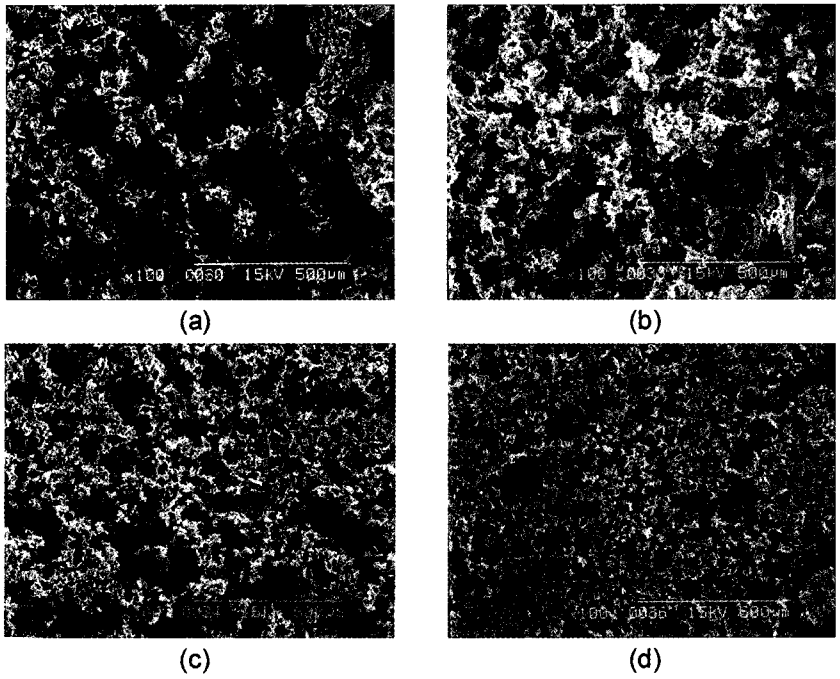

Figure 5. SEM microphotographs of DBP/PLA scaffolds by means of the emulsion freeze drying method (original magnification, ×100); (a) PLA, (b) 20% DBP, (c) 40% DBP, and (d) 80% DBP.

SEM Analysis of Pore Structure. SEM microphotographs of DBP/PLA scaffolds with control, 20, 40 and 80% DBP by means of emulsion freeze drying method are shown in Figure 5. All samples were highly porous with good interconnections between pores in which can support the surface of cell growth, proliferation and differentiation. To create an emulsion from two immiscible phases composed of PLA/MC phase and water/DBP phase, the control of various processing factors such as volume fraction of the dispersed phase, polymer concentration, and so on are very important.[41] Also, the effect of emulsion stability on scaffolds pore structure must be counted on because emulsions always tend to unstable system, that is to say, tend to separate immiscible phases resulting in poor dispersed pore property. Too stabilized emulsion, however, for example, fine particles as 1 μm or submicron sized emulsion might be not good for pore size for the application

of tissue engineering since cells can not be crawling into too small size pore. Physical properties and forces involved in emulsion stability include the interfacial energy,[42] the relative viscosity increment,[43] gravity,[44] the diffusion coefficient of the dispersed particles, the height of the free energy barrier preventing coagulation, and the electrical repulsive force between particles.[45] It can be observed that the pore size decreased in the order of 79 μm (PLA control) > 47 μm (20% of DBP) > 23 μm (40% of DBP) > 15 μm (80% of DBP) as listed in Table I. Porosities as well as specific pore areas decreased from 91% (PLA control) to 77% (80% of DBP) and 43 m^2/g (PLA control) to 11 m^2/g (80% of DBP), respectively, with increasing the amount of DBP. It can be explained that DBP act like emulsifier resulting in stabilizing water droplet in emulsion.[41] These stabilizing water droplets were tended to decreasing pore size in organic phase. Of course, the pore size can be controlled another factors as polymer concentration and so on to get lager pore size. Another possible explanation might be that increased DBP powder hinder the reassemble of water droplet in organic phase resulting in the smaller pore diameter.

SEM microphotographs of DBP/PLA scaffolds with control, 20, 40 and 80% DBP by means of solvent casting/salt leaching method are shown in Figure 6 and 7. 20 w/v% of PLA concentration

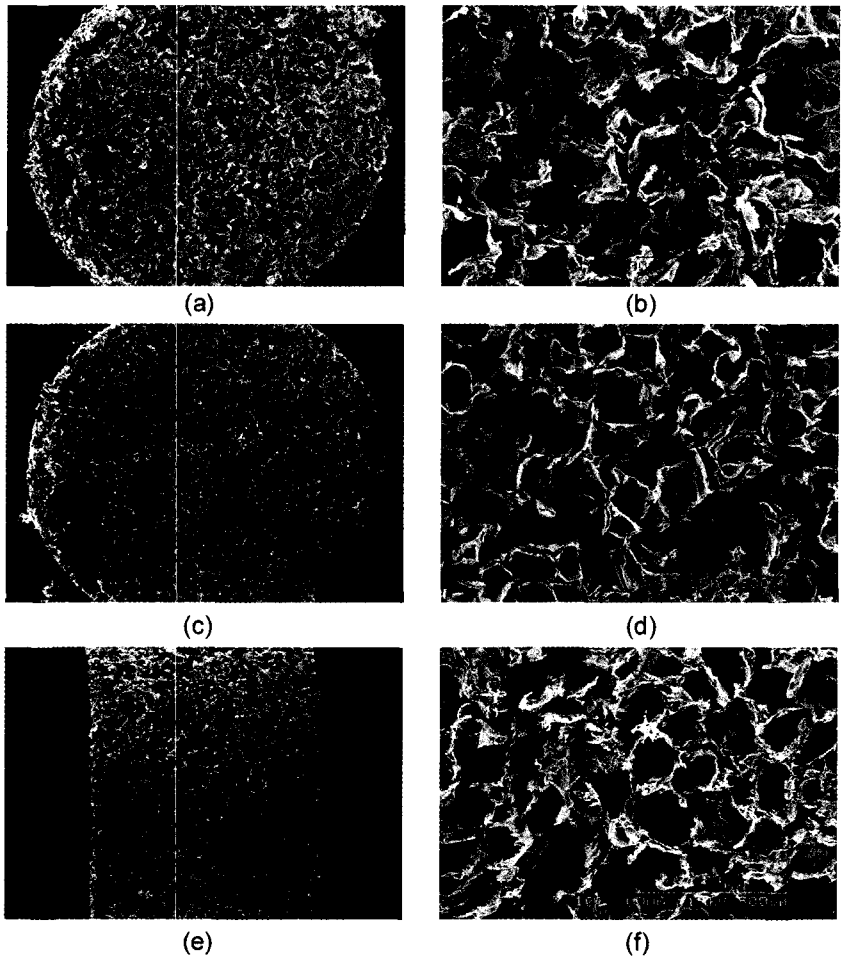

Figure 6. SEM microphotographs of DBP/PLA scaffolds 40% of DBP by means of the solvent casting/salt leaching method; (a) surface ×30, (b) surface ×100, (c) cross × 30, (d) cross ×100, (e) side ×30, and (f) side ×100.

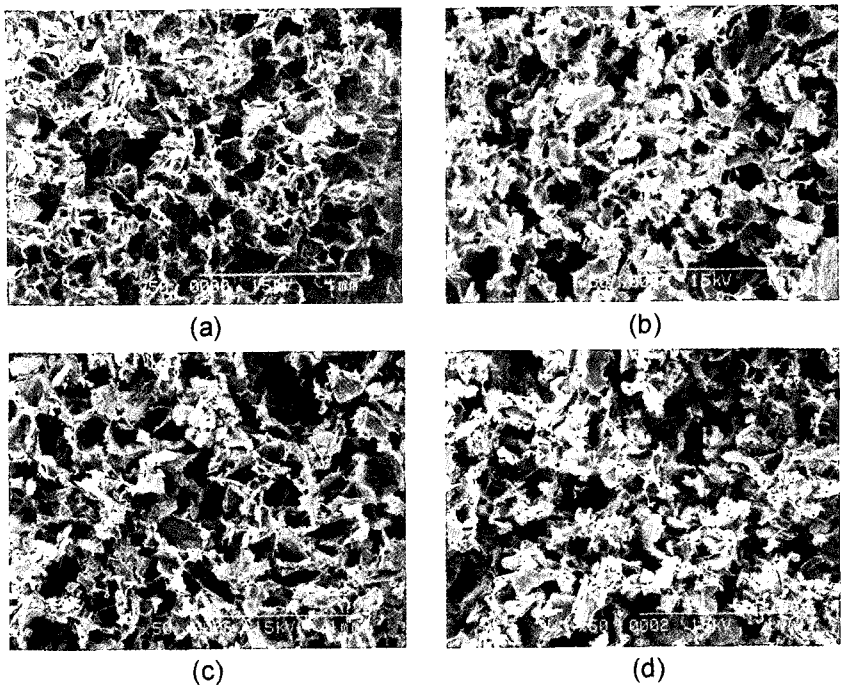

Figure 7. SEM microphotographs of DBP/PLA scaffolds by means of the solvent casting/salt leaching method (original magnification, ×50); (a) PLA, (b) 20% DBP, (c) 40% DBP, and (d) 80% DBP.

and 90 w/w% of PLA to ammonium bicarbonate were fixed and then DBP content were varied 20, 40 and 80 w/w% to PLA. All of surface, cross section, and side of DBP/PLA scaffolds were highly porous with good interconnections between pores in which can support the surface of cell growth, proliferation and differentiation similar with observed in Figure 5. Particularly, a uniform distribution of well interconnected pores from the surface to core region. It can be observed that the pore size was almost same, however, in the order of 81 μm (PLA control) $\geqq$ 76 μm (20% of DBP) $\geqq$ 74 μm (40% of DBP) > 69 μm (80% of DBP) as listed in Table II. Porosities as well as specific pore areas also were almost same from 97.8 % of PLA control to 94.4 % of 80% DBP and 68.3 m^2/g of PLA control to 62.75 m^2/g of 80% DBP, respectively, even though the amount of DBP increased.

For pore size distribution, Figure 8 and 9 show the results of mercury intrusion porosimeter analysis for the emulsion freeze drying method and the solvent casting/salt leaching method, respectively. The pore size distribution of the emulsion freeze drying method was broader than that of the solvent casting/salt leaching method independent with DBP content. It can be explained that the size distribution of water droplet was very broad in organic phase generated by ultrasonication as explained earlier. However, the size distribution of the solvent casting/salt leaching method was con-

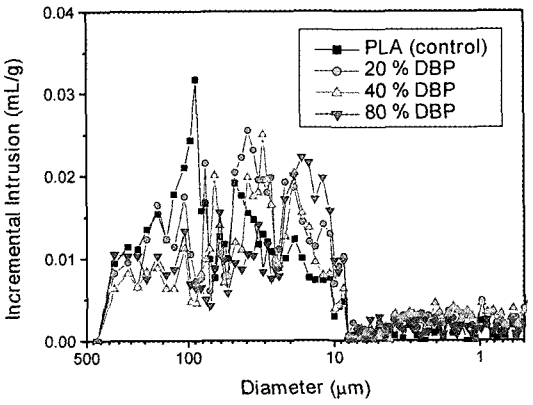

Figure 8. Pore size distribution of DBP/PLA scaffolds by means of the emulsion freeze drying method.

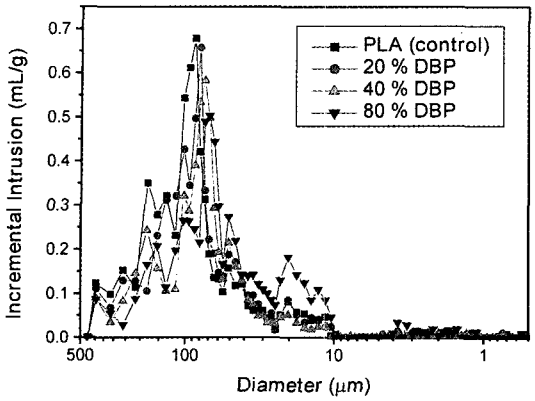

Figure 9. Pore size distribution of DBP/PLA scaffolds by means of the solvent casting/salt leaching method.

stantly uniform since the size distribution of a porogen as ammonium bicarbonate was also same.

The physical and chemical requirements of scaffolds for cell/tissue ingrowth are[46] (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). The preparation techniques for DBP/PLA scaffolds between the emulsion freeze drying and the solvent casting/salt leaching methods have been compared in terms of the scaffolds macrostructure and above physical and chemical requirements. For macrostructure of DBP/PLA scaffolds, the solvent casting/salt leaching methods might be more recommended due to 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 DBP contents, and less processing variables compared with the emulsion freeze drying. From the base of these results, we decided that the animal experiments are carrying out using the series of DBP/PLA scaffold prepared by the solvent casting/salt leaching methods.

These two techniques, even though the pore properties of the emulsion freeze drying method was little lower than that of the solvent casting/salt leaching methods, were demonstrated to be capable of preparing scaffolds of novel DBP/PLA as natural/synthetic composites with wide ranges of porosity, median pore size, pore size distribution, specific pore area, and good interconnection between pores.[41] These scaffolds possess relatively good physical properties and can be easily handled. The advantage of the emulsion freeze drying method is the possibility of the controlled bioactive molecule delivery system due to no leaching process resulting in loosing of water soluble bioactive molecule into water.[38] Also, the thick scaffold above 1 cm thickness can be prepared from the emulsion freeze drying method compared with other methods.[41] Therefore, we must choose that the appropriate scaffolds prepared various methods fitted the physical and chemical properties will be applied tissue and organ. In summary, these scaffolds prepared from these methods should be very useful in the application for tissue regeneration and reconstruction.

Implantation of DBP/PLA Scaffolds. The scaffolds fabricated by the solvent casting/salt leaching methods will be utilized to transplant and applied as a template guiding the formation of a bone and cartilage structures from the induction of osteogenesis and chondrogenesis. Scaffolds of PLA alone, DBP/PLA of 40 and 80%, and DBP powder were implanted on the back of athymic nude mouse to observe the effect of DBP on the induction of cells proliferation for 8 weeks. In PLA scaffold (Figure 10(A)), fibroblast-like cells (f) and vascular capillary (v) were observed between pore interconnection of the undegradaded PLA (l), however, there was no evidence of new bone formation. In DBP powder implants (Figure 10(B)), we can observe the evidence of calcification (c) around DBP (bp) from the undifferentiated stem cells in the subcutaneous sites and other soft connective tissue sites having a preponderance of stem cells.[28-30] It might be suggested that bone morphogenetic protein in the DBP stimulate osteogenesis. Urist[26,27] explained by the experiment of subcutaneous implant in allogenic recipients

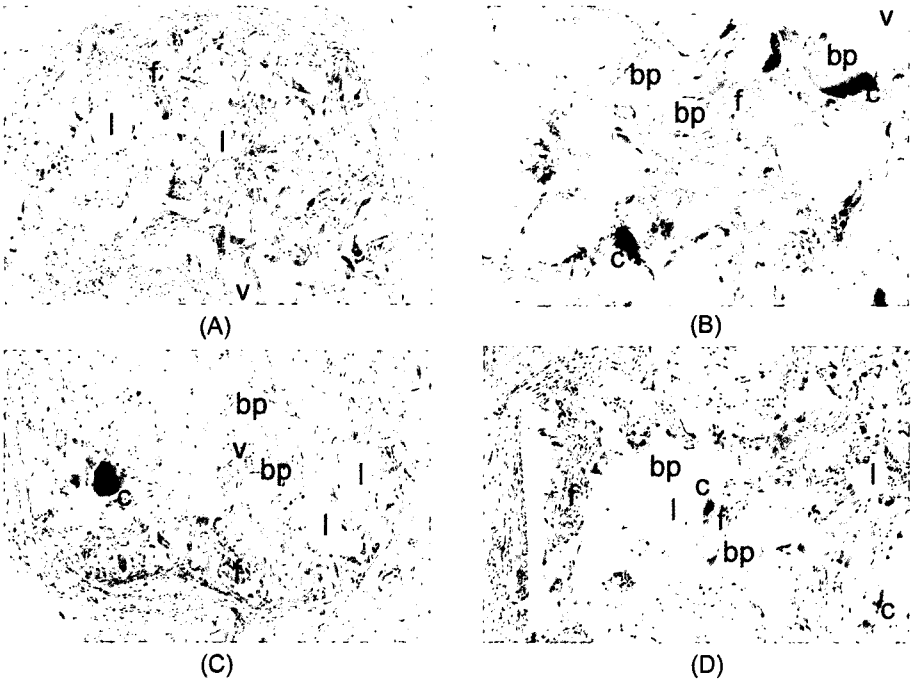

Figure 10. Photomicrographs from H & E histological sections of implanted (A) PLA scaffold, (B) DBP powder, (C) DBP/PLA scaffolds with 40% of DBP, and (D) DBP/PLA scaffolds with 80% of DBP.

that by day 5, mesenchymal stem cells differentiate, and by day 7 chondroblast developed with osteoblasts appearing at day 9. Angiogenesis ensues around day 11, and this correlates with chondrolysis. New bone formation occurs between 12 and 18 days postimplantation. Finally, ossicle development replete with hematopoietic marrow lineage occurs by day 21.

In DBP/PLA scaffolds of 40 and 80% DBP (Figure 10(C) and 10(D), respectively), the evidence of calcified region (c) around DBP (bp) as well as fibroblast-like cells (f) and vascular capillary (v) between pore interconnection of the undegradaded PLA (l) were observed. We conclude that the effect of DBP/PLA scaffolds on bone induction are stronger than PLA scaffolds, even though the bone induction effect of DBP/PLA scaffold might be lowered than only DBP powder, that is to say, in the order of DBP only > DBP/PLA scaffolds of 40 and 80% DBP > PLA scaffolds only for osteoinduction and chondroinduction activity. In summary, it seems that DBP plays an important role for bone and cartilage induction in DBP/PLA scaffolds.

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

Acknowledgments. This work was supported by grant from KRF 2000-F00268.

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