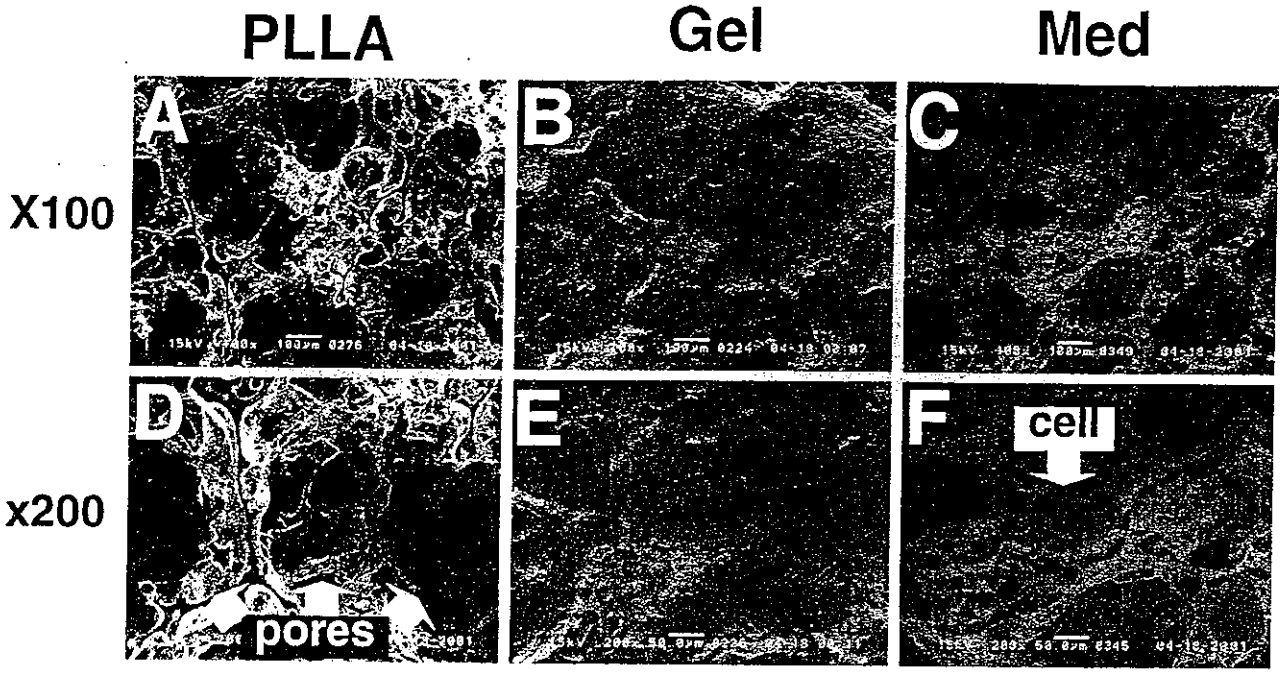

Figure 3. Inner surface observation of artificial vascular grafts. (A) Only PLLA vascular graft (original magnification ×100). (B) PLLA vascular graft with SMCs and collagen gel (original magnification ×100, our original model). (C) PLLA vascular graft inoculated SMCs as cell suspensions (original magnification ×100, a conventional model). (D) Only PLLA vascular graft (original magnification ×200). (E) PLLA vascular graft with SMCs and collagen gel (original magnification ×200, our original model). (F) PLLA vascular graft inoculated SMCs as cell suspensions (original magnification ×200, a conventional model).

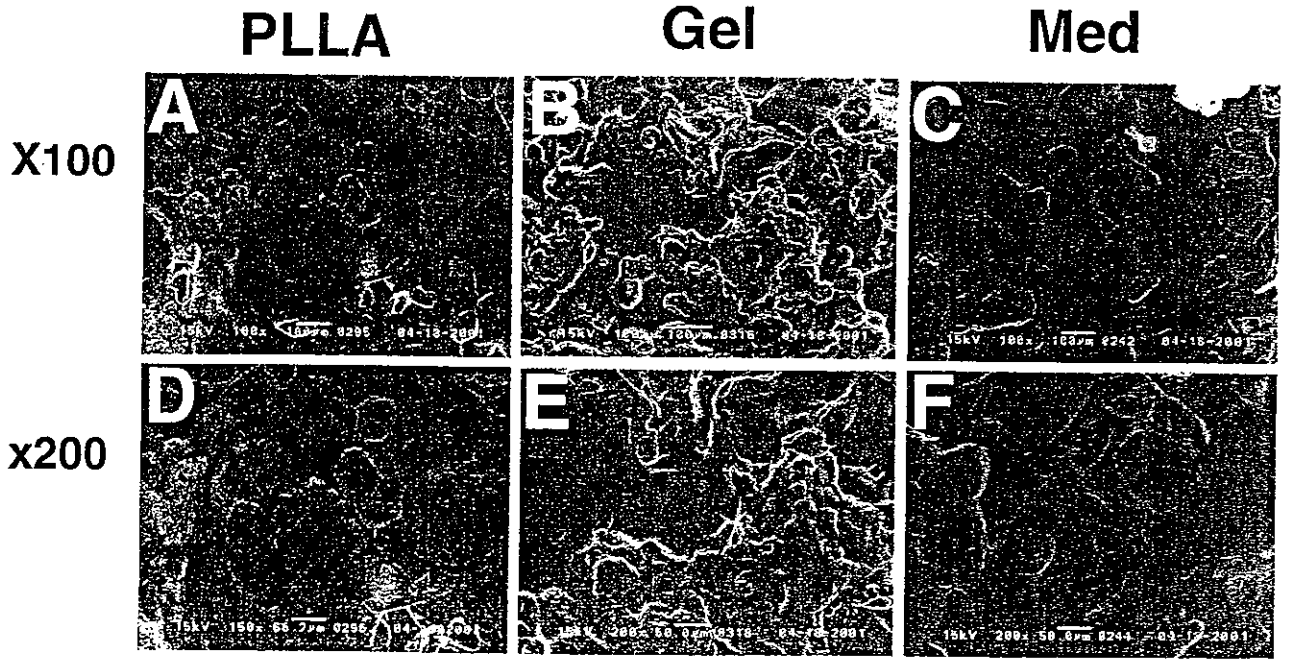

Figure 4. Outer surface observation of artificial vascular grafts. (A) Only PLLA vascular graft (original magnification ×100). (B) PLLA vascular graft with SMCs and collagen gel (original magnification ×100, our original model). (C) PLLA vascular graft inoculated SMCs as cell suspensions (original magnification ×100, a conventional model). (D) Only PLLA vascular graft (original magnification ×200). (E) PLLA vascular graft with SMCs and collagen gel (original magnification ×200, our original model). (F) PLLA vascular graft inoculated SMCs as cell suspensions (original magnification ×200, a conventional model).

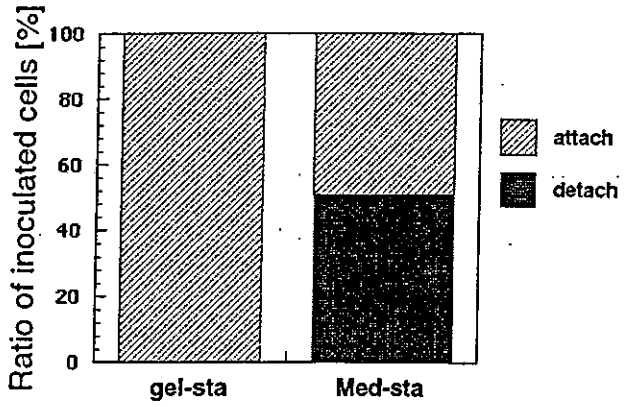

Figure 5. Efficiency of inoculation of cells into the artificial vascular grafts. (A) Our new model ($n = 4$). (B) A conventional model ($n = 3$).

already been reported, but there were drawbacks such as weak mechanical properties. In contrast, a higher encapsulation ratio of cells to gel was achieved in the artificial vascular grafts, and smooth inner surfaces were rapidly formed. Therefore, our new model could be understood as a complementary model. There was a significant difference between the ratio of trapped SMCs to those inoculated by using collagen solution (our new model) and that by medium (conventional model). Rapid formation of a smooth inner surface and the development of artificial vascular grafts with suitable mechanical properties became simultaneously possible in our model.

To construct the artificial vascular graft model with gel and a biodegradable polymer, we selected collagen gel and PLLA, but this is merely one example. In this article we showed our concept of building an artificial vascular graft by using collagen gel and a PLLA scaffold. In the next step, other materials such as polyglicolic acid, poly-lactic acid–glicolic acid copolymer, and so on, should be used in an attempt to make an artificial vascular graft. A further experimental design of an artificial vascular graft taking into account degradation speed and the biocompatibility of materials would be needed.

Our new model has two ways to endothelialize the surface of the artificial vascular graft in vivo and in vitro. In the in vivo model, SMCs inoculated into the artificial vascular graft would induce prompt endothelialization and contribute to the adhesion and stay of endothelial cells on the inner surface. In addition, a smooth inner surface would avoid thrombogenesis, because a rough surface leads to turbulent flow. Consequently, the ratio of the patency increases after implantation. On the other hand, a model inoculating endothelial cells in vitro attains a stable nonthrombogenic environment before implantation, because endothelial cells release anticoagulant and antithrombogenic factors onto the inner surface exposed to blood. It has already been reported that the functions of endothelial cells secreting those factors are enhanced in accordance with increases in shear stresses caused by blood flow in vivo (2,15). Therefore, it is understood that the endothelial cells regulate the antithrombogenic environment in vivo. It is crucial to inoculate previously endothelial cells into the artificial vascular graft, but the preparation and the culture of endothelial cells are chargeable. Therefore, according to the diameter size of the artificial vascular grafts, manifestation of patents, a suitable model should be chosen. It is thought that further work on these points is required.

The inclusion of cells is needed to reconstitute an artificial vascular graft, because the inoculated cells induce and stimulate the remodeling of themselves. In our model incorporating a PLLA scaffold and gel, almost all SMCs inoculated into the artificial vascular graft were trapped, whereas a lot of SMCs were detached from the PLLA scaffold in a conventional model inoculating cells as a cell–medium suspension. Therefore, it was thought that our model induces easily remodeling of the artificial vascular grafts.

In conclusion, the results of the experiments described above suggested that our model as an artificial vascular graft was a useful one. Furthermore, our method would be able to be applied to other tissues such as an artificial nervous system, an artificial bone, etc.

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Three-Dimensional Seeding of Chondrocytes Encapsulated in Collagen Gel Into PLLA Scaffolds

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Tissue engineering approaches have been clinically tried to repair damaged articular cartilages. It is an essential step to uniformly seed chondrocytes into 3D scaffolds in order to reconstruct tissue-engineered cartilages in vitro, but the tissue engineering could not have been provided with efficient cell seeding methods. Type I collagen is clinically used and known as a cytocompatible material, having recognition sites for integrins. Collagen gel encapsulating chondrocytes has been tried for making regenerated cartilages, but it is found difficult to have the gel keep its original shape after long-term culture, because of shrinking. On the other hand, 3D scaffolds, either of a nonwoven structure or a sponge-like structure, involve difficulty in that chondrocytes could not be uniformly seeded, although they have adequate initial mechanical properties. In this study, by combining collagen gelation with a nonwoven PLLA scaffold, we achieved uniform cell seeding into the 3D scaffold. Bovine articular chondrocytes were mixed with type I collagen solution, and the solution was poured into the nonwoven PLLA scaffold (1.5 mm thick, ϕ 15 mm). The collagen–chondrocyte mixture was made into gel at 37°C for 1 h. The 0.39% collagen mixture was viscous enough to prevent cells from precipitating during gelation. Almost all chondrocytes were able to be incorporated into the PLLA scaffolds by mixing with collagen solution and subsequently making into gel, while 30–40% of the chondrocytes seeded as a cell suspension were not trapped into the PLLA scaffolds. The method presented, where chondrocytes were mixed with collagen solution, and the mixture was incorporated into a 3D scaffold, then made into gel in the scaffold, could serve as an alternative for in vitro cartilage regeneration, also simultaneously having the advantages of both materials.

Key words: Chondrocyte; Collagen; Gelation; PLLA; Cartilage; Regeneration

INTRODUCTION

Articular cartilage is rarely repaired by itself. Therefore, a variety of clinical attempts have been tried, including tissue engineering approaches. (9,12,20,24). In order to reconstruct three-dimensional cartilage-like tissues, diverse kinds of scaffolds have been employed. Some of the materials were nonbiodegradable such as silicone rubber (2), polytetrafluoroethylene (PTFE), polyethylene terephthalate (Dacron) (18,19), polyurethanes (16), poly(hydroxyethyl methacrylate) (PHEMA) (17), and polyvinyl alcohol (PVA) (8). On the other hand, natural biomaterials or biodegradable synthetic polymers have also been used for cartilage regeneration: collagen sponges and gels (20) and hyaluronan (1,22) as natural ones, and polyglycolide (PGA) (13), polylactide (PLA) (7), poly(lactide-*co*-glycolide) (PLGA) (23), poly(L-lactide-*co*-caprolactone) (L-PLCL) (14), and poly(glycolide-*co*-trimethylene carbonate) (PGTMC) (11) as synthetic ones.

The biodegradable polymers approved by the FDA, such as PLLA, PGA, and PLGA, are easily processed into desired shapes with good mechanical properties. Their degradation periods can be manipulated by controlling their crystallinity, molecular weight, and copolymer ratio of lactic acid to glycolic acid, but PLLA-, PGA-, and PLGA-derived scaffolds lack cell recognition signals, and their hydrophobic property hinders smooth cell seeding. On the other hand, naturally derived collagen has the potential advantage of specific cell interaction, although it offers limited versatility in designing a scaffold with specific physical properties (10,15,21). By using the advantages of both materials, we have developed hybrid sponges of PLGA and collagen, fabricated by forming microsponges of collagen in the pores of a PLGA sponge for cartilage regeneration (3–5) and for skin regeneration (6). This sponge is hydrophilic with good mechanical strength, but it is still difficult to seed cells in deep areas of the scaffolds through the pores. In this study, by combining collagen gelation with a non-

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woven poly-L-lactic acid (PLLA) scaffold, we achieved uniform cell seeding into the 3D scaffold.

MATERIALS AND METHODS

Materials

PLLA nonwoven fiber scaffolds were made so that the thickness was 1.5 mm (density: 0.13 g/ml) (Fig. 1). Collagen gel was purchased from Koken (Japan). Minimum essential medium Ham's F-12 was purchased from Gibco BRL Laboratory (Grand Island, NY), fluorescent dye 4,6-diamidino-2-phenylindole (DAPI) from Wako Pure Chemical Industries, and safranin-O and toluidine blue from Chroma-Gesell Schaft (Germany).

Bovine Articular Chondrocyte Culture

Bovine articular cartilage chondrocytes were obtained from the knee joints of young calves (6–12 months old). The cartilage was dissected, minced, and digested in a collagenase solution (Worthington Biochemical Cooperation, NJ). After overnight digestion, the cell suspension was filtered with a 70-μm cell strainer (Falcon, Becton Dickinson, NJ). The filtered solution was centrifuged to remove the collagenase solution. The cells were resuspended in Ham's F-12 medium and seeded in 75-cm^2 culture flasks (Falcon). The cells were used for the study after 3–5 passages.

Collagen–Chondrocyte Mixture

Five hundred micrometers of 0.39% or 0.18% collagen solution with 50 μm of sodium bicarbonate (3.7 g/L) and 10 μm of 1 M HEPES was mixed together with bovine chondrocytes so that the cell concentration became 6×10^6 cells/ml.

Cell Seeding to PLLA Scaffolds

The PLLA scaffolds were cylindrically cut so that the diameter was 15 mm (1.5 mm in thickness). The following five kinds of PLLA–chondrocyte constructs were made. Each construct was incubated with F-12 10% FBS in the well of six-well plates (Falcon) at 37°C in a humidified 5% CO_2 atmosphere.

1. PLLA: 1000 μl of chondrocyte suspension (3.0 and 6.0×10^6 cells/ml) was poured onto each PLLA scaffold prepared.
2. PLLA-hy: The PLLA scaffolds were treated sequentially with 75% and 100% EtOH solution (1 h). 1000 μl of chondrocyte suspension (3.0 and 6.0×10^6 cells/ml) was poured onto each PLLA scaffold treated.
3. PLLA-coated: The PLLA scaffolds were coated with 0.1% collagen solution (1 h). 1000 μl of chondrocyte suspension (3.0 and 6.0×10^6 cells/ml) was poured onto each PLLA scaffold coated.
4. PLLA-0.39Col: 500 μl of 0.39% collagen mixture containing chondrocytes (3.0 and 6.0×10^6 cells/ml) was incorporated into each PLLA scaffold. The incorporated collagen mixture was made into gel by incubating at 37°C for 1 h.
5. PLLA-0.18Col: 500 μl of 0.18% collagen mixture containing chondrocytes (3.0 and 6.0×10^6 cells/ml) was incorporated into each PLLA scaffold. The incorporated collagen mixture was made into gel by incubating at 37°C for 1 h.

Histochemical Analysis

After fixation of the constructs with 4% formalin/PBS solution, sliced samples were made by cutting the constructs into 8 μm thickness with a cryostat (CM1850, Leiz, Germany). The nuclei and extracellular matrices of the samples were stained with Hoechst 33342, safranin-O, and toluidine blue, respectively. The histochemical observation of the samples was made with an inverted epifluorescence microscope (Axiovert 100-M, Zeiss, Germany).

RESULTS

Figure 2 shows the nucleus staining with Hoechst 33342 of seeded chondrocytes into five kinds of con-

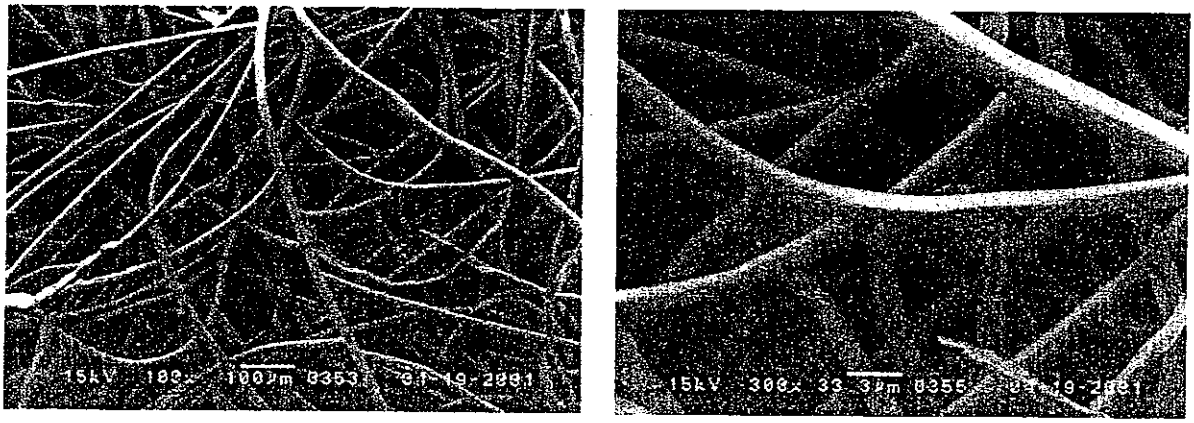

Figure 1. SEM observation of a nonwoven PLLA scaffold used in this study.

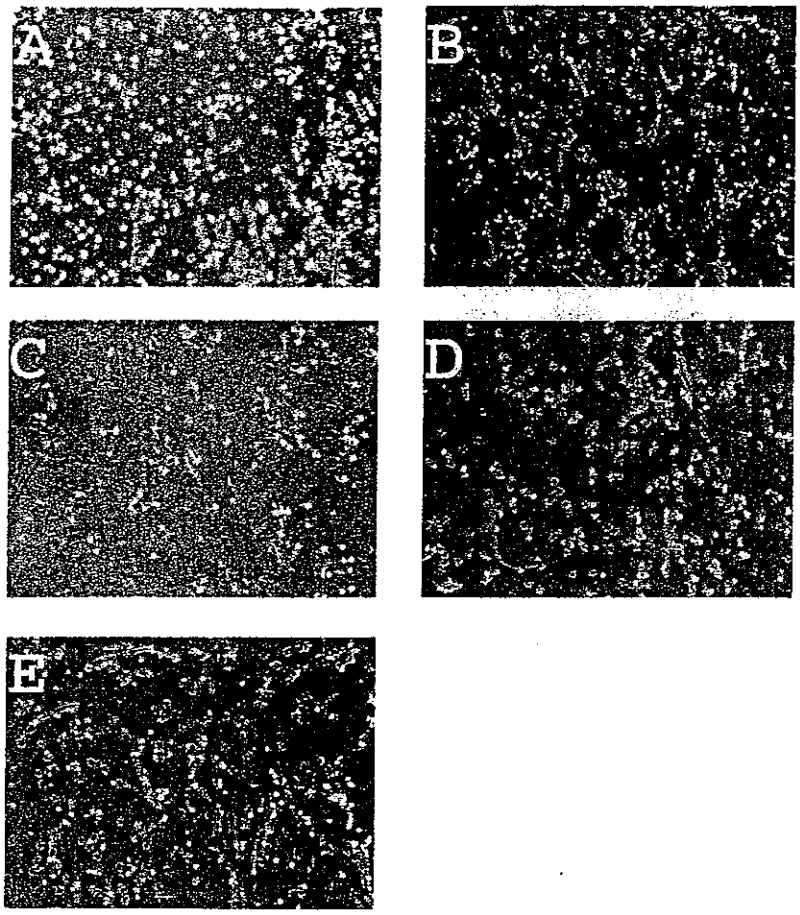

Figure 2. Nucleus staining of chondrocytes seeded into a PLLA nonwoven scaffold. (A) PLLA-0.39Col (chondrocytes seeded with 0.39% collagen solution mixture, 6×10^6 cells/ml). (B) PLLA-0.18Col (chondrocytes seeded with 0.18% collagen solution mixture, 6×10^6 cells/ml). (C) PLLA-hy (chondrocytes seeded as a suspension, 6×10^6 cells/ml, into the nonwoven PLLA scaffold treated with EtOH). (D) PLLA-coated (chondrocytes seeded as a suspension, 6×10^6 cells/ml, into the nonwoven PLLA scaffold coated with type I collagen). (E) PLLA (chondrocytes seeded as a suspension, 6×10^6 cells/ml, into the nonwoven PLLA scaffold).

structs after 1 day of culture. The figure shows that chondrocytes mixed with collagen solution were homogeneously distributed into the PLLA scaffolds. The chondrocytes seeded as a cell suspension were found to be trapped by adhering to the PLLA fibers.

Figure 3 shows the ratio of untrapped chondrocytes to those seeded into the scaffolds. The number of untrapped chondrocytes was evaluated by measuring the fluorescence intensity of DNA stained with Hoechst 33342. The figure shows that almost all chondrocytes could be incorporated into the PLLA scaffolds by mixing with collagen solution and subsequently making into a gel, although 30–40% of chondrocytes seeded as a cell suspension were not trapped into the PLLA scaffolds.

Figure 4 shows the difference in distribution homogeneity of chondrocytes incorporated with 0.10%, 0.18%, and 0.39% of collagen solutions into the PLLA scaffolds. The chondrocytes mixed with 0.10% or 0.18% collagen solution were partially precipitated during gelation, while chondrocyte precipitation was not detected in the case of 0.39% collagen solution.

Figure 5 shows safranin-O stain and toluidine blue stain of the sliced constructs after 9 days of culture. The safranin-O and toluidine blue stains show that both chondrocytes in collagen gel and those adhering to the fibers were stained with safranin-O and toluidine blue, although matrices produced by seeded chondrocytes were not apparently detected after 9 days of culture.

DISCUSSION

The PLLA scaffolds used in the experiments were made of nonwoven PLLA fibers as shown in Figure 1. The felt-like structure of the scaffolds is thought to allow seeded cells to access relatively deeper sites com-

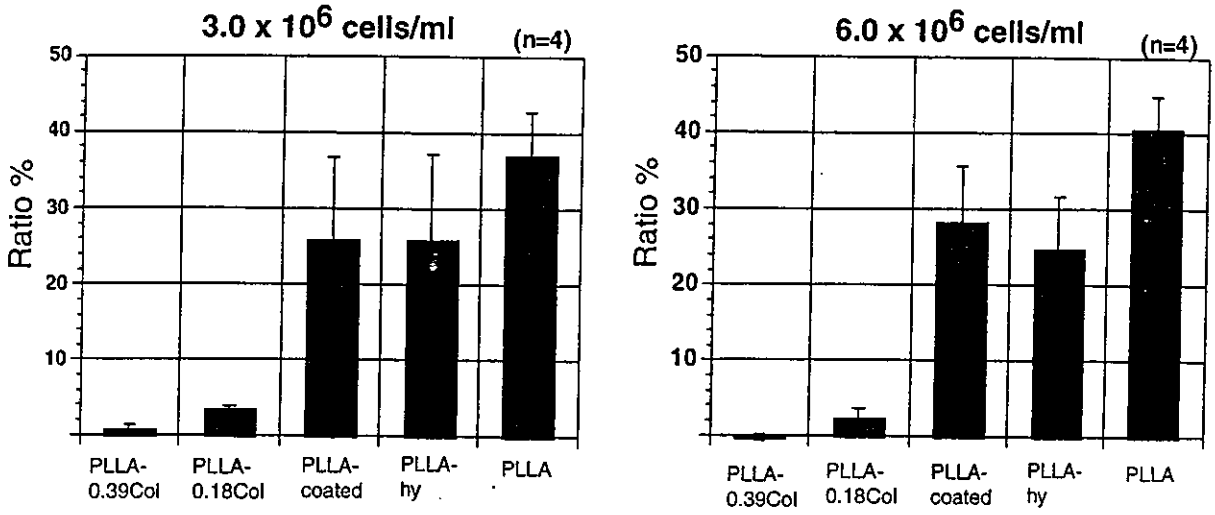

Figure 3. Ratio of untrapped chondrocytes to those seeded into the scaffolds.

pared with a sponge-like structure. As shown representatively in Figure 2, the chondrocytes seeded as a cell suspension could adhere to the fibers in all areas of sliced sections. The thickness (1.5 mm) of the scaffolds might facilitate cell access even to the center area of the scaffolds. As the thickness of articular cartilage is equivalent to that of the scaffolds, the PLLA scaffolds used could serve as adequate ones for cartilage regeneration. On the other hand, both 0.18% and 0.39% collagen mixtures with chondrocytes could penetrate the scaffolds without any physical supports. After making into gel at 37°C for 1 h, the chondrocytes were still uniformly distributed in the gel in the case of 0.39% collagen mixture, whereas slight precipitation of chondrocytes was detected in the case of 0.10% or 0.18% collagen mixture. This means that 0.39% collagen mixture was viscous enough to prevent the cells from precipitating during gelation.

Figure 3 shows that 30–40% of chondrocytes passed through the scaffolds without adhering to the fibers. As PLLA is known to be hydrophobic, EtOH treatment or 0.1% collagen coating was tried to improve the hydrophobicity. However, no significant difference was found in the ratio of untrapped chondrocytes to those seeded among no treatment, EtOH treatment, and 0.1% collagen coating, as shown in Figure 3. In the case of collagen mixtures, almost no leakage of chondrocytes from the gel was detected. This means that the method by which cells were incorporated into scaffolds with the collagen solution mixture was superior to the conventional method using a cell suspension from the point of view of seeding efficiency.

For in vitro cartilage regeneration, the following two critical points are emphasized: one is how to seed chondrocytes into scaffolds without any heterogeneous areas from a point of chondrocyte distribution, and the other is how to control the 3D shape of constructs and maintain their mechanical properties. Collagen gel encapsulating chondrocytes has been tried for that purpose. However, it was found difficult to maintain the original shape of the gel after long-term culture, because of its shrinking. On the other hand, 3D scaffolds, whether of

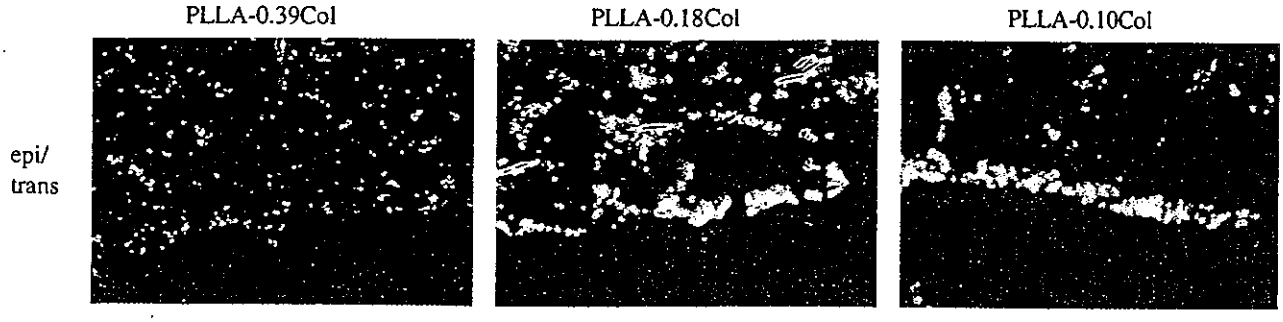

Figure 4. Difference in distribution homogeneity of chondrocytes incorporated with 0.10%, 0.18%, and 0.39% of collagen solutions into the PLLA scaffolds (6×10^6 cells/ml for 9 days).

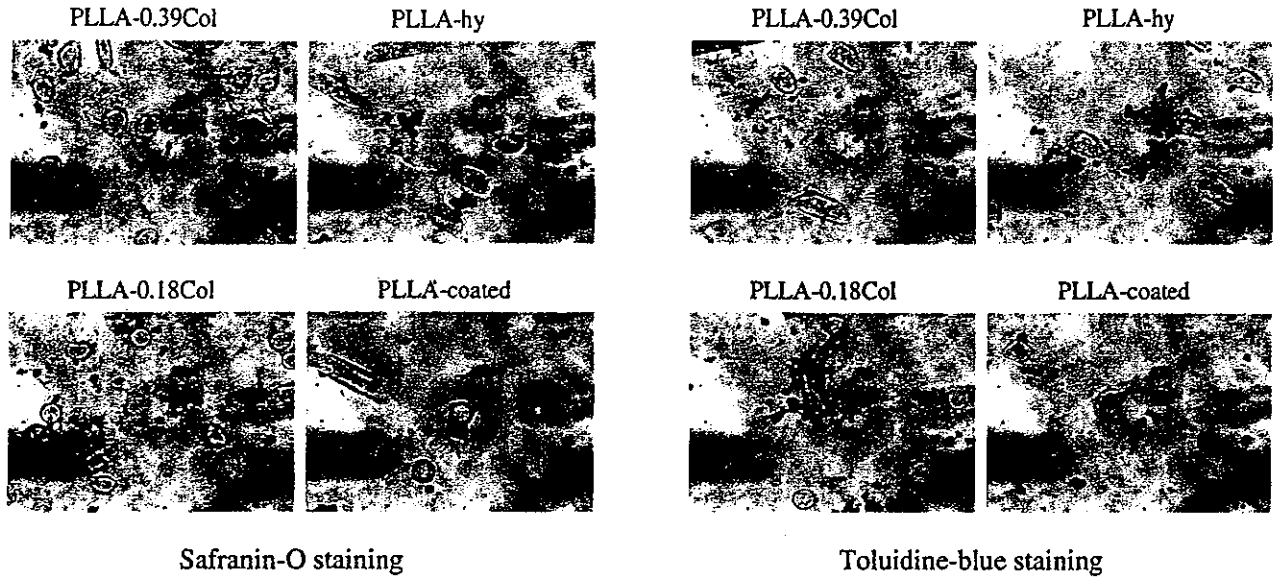

Figure 5. Safranin-O stain and toluidine blue stain of the sliced constructs after 9 days of culture (6×10^6 cells/ml).

a nonwoven structure or a sponge-like structure, present a difficulty in uniformly seeding chondrocytes, although they have adequate initial mechanical properties. We have developed a PLGA sponge nested with collagen microsponges, where collagen microsponges were made in the macropores of the PLGA sponge (3–5). This scaffold incorporated the most desirable properties of each material: collagen as a cytocompatible material and PLGA as a biodegradable one with good mechanical properties. The method presented here, where chondrocytes were mixed with collagen solution, and the mixture was incorporated into a 3D scaffold, then made into gel in the scaffold, could serve as an alternative one for in vitro cartilage regeneration, also simultaneously taking advantages of the properties of both materials.

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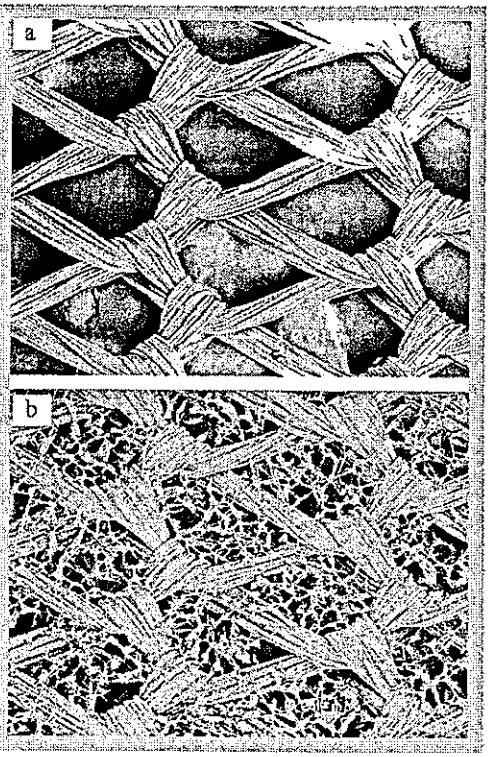
Review: Tissue engineering has emerged as a promising alternative approach in the treatment of malfunctioning or lost organs. In this approach, a temporary scaffold is needed to serve as an adhesive substrate for the implanted cells and a physical support to guide the formation of the new organs. In addition to facilitating cell adhesion, promoting cell growth, and allowing the retention of differentiated cell functions, the scaffold should be biocompatible, biodegradable, highly porous with a large surface/volume ratio, mechanically strong, and malleable. A number of three-dimensional porous scaffolds fabricated from various kinds of biodegradable materials have been developed. This paper reviews some of the advances in scaffold design focusing on the hybrid scaffolds recently developed in the authors' laboratory.



SEM photomicrographs of PLGA knitted mesh (a) and PLGA–collagen hybrid mesh (b).

Scaffold Design for Tissue Engineering

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Introduction

Organ and tissue loss or failure resulting from an injury or other type of damage is a major human health problem. Tissue or organ transplantation is a standard therapy to treat these patients, but this is severely limited by donor shortage. Other available therapies including surgical reconstruction, drug therapy, synthetic prostheses, and medical devices are not limited by supply, but they do have other problems. For example, synthetic prostheses and medical devices are not able to replace all the functions of a damaged or lost organ or tissue. The efforts to address these problems and limitations have elicited the development of new biomaterials and alternative therapies.

Tissue engineering has emerged as a promising alternative approach to treat the loss or malfunction of a tissue or organ without the limitations of current therapies.[1–7] Tissue engineering involves the expansion of cells from a small biopsy, followed by the culturing of the cells in temporary three-dimensional scaffolds to form the new organ or tissue. By using the patient's own cells, this approach has the advantages of autografts, but without the problems associated with adequate supply.

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With this approach, porous three-dimensional temporary scaffolds play an important role in manipulating cell function and guidance of new organ formation.[8–12] Isolated and expanded cells adhere to the temporary scaffold in all three dimensions, proliferate, and secrete their own extracellular matrices, replacing the biodegrading scaffold. Significant challenges to this approach include the design and fabrication of the scaffolds. Ideally, scaffolds for tissue engineering should meet several design criteria: (1) the surface should permit cell adhesion, promote cell growth, and allow the retention of differentiated cell functions; (2) the scaffolds should be biocompatible, neither the polymer nor its degradation by-products should provoke inflammation or toxicity in vivo; (3) the scaffold should be biodegradable and eventually eliminated; (4) the porosity should be high enough to provide sufficient space for cell adhesion, extracellular matrix regeneration, and minimal diffusional constraints during culture, and the pore structure should allow even spatial cell distribution throughout the scaffold to facilitate homogeneous tissue formation; (5) the material should be reproducibly processable into three-dimensional structure, and mechanically strong. A number of three-dimensional porous scaffolds fabricated from various kinds of biodegradable materials have been developed and used for tissue engineering of liver,[13] bladder,[14,15] nerve,[16] skin,[17] bone,[18] cartilage,[19–22] and ligament,[23,24] etc. This review summarizes some of the recent developments.

Biomaterials Applied for Tissue Engineering

Over the last century, biocompatible materials such as metals, ceramics and polymers have been extensively

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used for surgical implantation. Metals and ceramics have contributed to major advances in the medical field, particularly in orthopaedic tissue replacement. However, metals and ceramics are not biodegradable and their processability is very limited. Polymer materials have received increasing attention and been widely used for tissue engineering because of easy control over biodegradability and processability.

There are two kinds of polymer materials: synthetic polymer, and naturally derived polymers.[10,25–30] The main biodegradable synthetic polymers include polyesters, polyanhydride, polyorthoester, polycaprolactone, polycarbonate, and polyfumarate.[31–38] The polyesters such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymer of poly[lactic-*co*-(glycolic acid)] (PLGA) are most commonly used for tissue engineering. They have gained the approval of the US Food and Drug Administration for certain human clinical use, such as surgical sutures and some implantable devices. PLA undergoes hydrolytic scission to its monomeric form, lactic acid, which is eliminated from the body by incorporation into the tricarboxylic acid cycle. The principal elimination path for lactic acid is respiration, and it is primarily excreted by lungs as CO_2. PGA can be broken down by hydrolysis, nonspecific esterases and carboxypeptidases. The glycolic acid monomer is either excreted in the urine or enters the tricarboxylic acid cycle.

The naturally derived polymers include proteins of natural extracellular matrices such as collagen and glycosaminoglycan, alginic acid, chitosan, and polypeptides, etc.[39–56] Yannas et al. conducted pioneering studies on collagen-glycosaminoglycan scaffolds to induce the regeneration of dermis of skin, sciatic nerve and knee meniscus.[57–60] Chemical crosslinking by glutaraldehyde has been proposed to control the stability and degradation rate of these matrices, whereas porosity has been changed by both chemical and physical techniques.[61,62] Alginic acid, a polysaccharide from seaweed, is a family of natural copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G). They have been processed in gel beads encapsulating living cells as a means of immunoprotection. Alginates crosslinked with calcium sulfate have been recently used as cell-delivery vehicles for tissue engineering, however gelation kinetics by the use of calcium sulfate is difficult to control, and the resulting structure is not uniform. Chitosan is a natural polysaccharide, whose structural characteristics are similar to glycosaminoglycans. It has been used in a variety of biomedical applications, such as hemodialysis membranes, drug delivery systems, orthopaedic and dental coating materials and artificial skin. Polypeptides with some amino acid sequences can be favorable to cell adhesion and function and thus they may have potential for cell attachment and transplantaion. The poor control of enzymatic degradation and poor mechanical performance are however two major objections to the use of naturally derived polymers.

Preparation of Porous Three-Dimensional Scaffolds

Porous three-dimensional scaffolds fabricated from synthetic and naturally derived biodegradable polymers such as PGA, PLA, PLGA, and collagen have been widely used in the tissue engineering of cartilage, bone, skin, and ligament, etc. Several methods have been developed to prepare these kinds of porous three-dimensional biodegradable scaffolds, including gas foaming,[63,64] fiber extrusion and bonding,[65] three-dimensional printing,[66] phase separation,[67–69] emulsion freeze-drying,[70] and porogen leaching.[71,72]

The gas-foaming technique uses high-pressure CO_2 gas processing. Macroporous sponges of PLLA, PLGA, and PGA have been fabricated by this method by equilibrating polymer discs with high pressure CO_2 gas. A polymer/gas solution is formed, creating pores. Thermodynamic instability is then generated by decreasing the CO_2 gas pressure. Gas molecules then cluster, forming nuclei, to minimize their free energy. Dissolved gas molecules diffuse to these pore nuclei, creating the macropores noted post processing. The porosity and pore structure is dependent on the amount of gas dissolved in the polymer, the rate and type of gas nucleation and the diffusion rate of gas molecules through the polymer to the pore nuclei. However, the gas-foaming technique often results in a closed cellular structure within the scaffold. To improve the pore structure, a combination technique of gas foaming and particulate leaching has been recently proposed.[64] Open-pore biodegradable scaffolds have been fabricated. Disks composed of biodegradable polymer and salt particulates are compression-molded at room temperature and subsequently allowed to equilibrate with high-pressure CO_2 gas. After expansion, the salt particulates are leached out to yield macropores within the polymer scaffold. The overall porosity and level of pore connectivity can be regulated by the ratio of polymer/salt particulates and the size of the salt particulates. More recently, macroporous biodegradable scaffolds have been fabricated using ammonium bicarbonate salt as a gas foaming agent as well as a porogen additive.[73,74] The scaffolds show macropore structures of over 200 μm with no visible surface skin layer. Porosities can be controlled by the amount of ammonium bicarbonate incorporated to the polymer.

Fiber meshes consist of individual fibers either woven or knitted into three-dimensional patterns of variable pore size. PGA has been the first biocompatible and degradable polymer to be spun into fibers and used as synthetic suture threads. Scaffolds shaped with PGA, PLGA, and PLLA fibers have been investigated for cell transplanta-

tion and regeneration of various tissues such as nerve,[75] skin,[76] esophagus,[77] ligament,[78] bladder,[79] and cartilage.[80-82] These fibers are also reinforcing agents of polymeric materials used in orthopaedic surgery. The advantages of fiber meshes are a large surface area for cell attachment and a rapid diffusion of nutrients in favor of cell survival and growth. The drawback of these scaffolds might be a lack of structural stability, which can be at least partly overcome by the hot-drawing of PLLA fibers which results in improved molecular orientation and crystallinity. Interconnected fiber networks have been reported by Mikos et al. using the so-called fiber bonding technique,[65] which involves the casting of a PLLA solution over a non-woven mesh of PGA fibers. Solvent evaporation results in a composite material which consists of non-bonded PGA fibers embedded in a PLLA matrix. Fiber bonding occurs during post treatment at a temperature above the melting temperature of PGA. Finally, the PLLA matrix is selectively dissolved in a non-solvent for PGA, and a network of bonded PGA fibers is released. Obviously, this technique is not most suitable for a fine control of porosity. An alternative method involves spraying a non-bonded PGA fiber mesh with an atomized solution of PLLA or PLGA.[83] The thickness of the polymer coating is controlled by the spraying time. The resulting composite material combines the mechanical properties of the PGA core and the surface properties of the PLLA or PLGA coating which are essential to cell attachment, growth and function.

Three-dimensional (3D) printing is a solid free-form fabrication process which produces components by ink-jet printing a binder into sequential powder layers.[66] The part is built sequentially in layers. The binder is delivered to the powder bed producing the first layer, the bed is then lowered to a fixed distance, powder is deposited and spread evenly across the bed, and a second layer is built. This is repeated until the entire part, e.g. a porous scaffold, is fabricated. Following treatment, the object is retrieved from the powder bed and excess unbound powder is removed. The speed, flow rate and even drop position can be computer controlled to produce complex 3D objects. The 3D printing technique appears useful, while its application for tissue engineering is still in infancy.

The phase-separation technique is based on thermodynamic demixing of a homogeneous polymer-solvent solution into a polymer-rich phase and a polymer-poor phase, usually by either exposure of the solution to another immiscible solvent or cooling the solution below a binodal solubility curve.[67-69] Solvent is removed by freeze-drying, leaving behind the polymer as a foam. Morphology is controlled by any phase transition that occurs during the cooling step, i.e., liquid–liquid or solid–liquid. It has been reported that the liquid–liquid phase separation leads to isotropic PLA forms with highly interconnected pores of 1–10 μm in diameter, the solid–liquid phase separation results in anisotropic foams with a sheet like morphology. Recently, macroporous open cellular scaffolds with pore diameters above 100 μm have been fabricated by controlling the coarsening process in the later stage of thermally induced phase separation.

The emulsion freeze-drying method has been used to fabricate aliphatic polyester-based scaffolds by Whang et al.[70, 84] Scaffolds with porosity greater than 90%, median pore sizes ranging from 15 to 35 μm, and with larger pores greater than 200 μm, have been prepared. The porosity and pore size depends on the volume fraction of the dispersed phase, polymer concentration and molecular weight. This technique has been devised for incorporating proteins into the polymer scaffolds. Freeze-drying of aqueous solutions of natural biopolymers such as collagen has been reported for the production of well-defined porous matrices (i.e., pore size and orientation) based on the controlled growth of ice crystals during the freeze-drying process.

The porogen leaching method involves the casting of a mixture of polymer solution and porogen in a mold, drying the mixture, followed by a leaching out of the porogen with water to generate the pores.[71, 72] Usually, water-soluble particulates such as salts and carbohydrates are used as the porogen materials. The pore structures can easily be manipulated by controlling the property and fraction of the porogen, and the process is reproducible. This technique provides easy control of the pore structure and has been well established. Highly porous PLLA membranes of controlled porosity, surface/volume ratio, and crystallinity have been prepared by casting dispersion of crystalline salt particles in PLLA organic solutions followed by the salt leaching with water once the solvent has been evaporated. Sodium chloride, tartrate and citrate of various sizes have been used as leachable particles. The foam properties depend only on the initial salt weight fraction and particle size, and are not affected by the salt type or solvent selection.

The phase separation and emulsion freeze-drying techniques can be devised for incorporating proteins into the scaffolds because the manipulations are conducted at low temperature.[84] However, the pore structures of the scaffolds cannot be easily controlled; also, it is difficult to obtain large, open pores. On the other hand, the porogen leaching method provides easy control of pore structure and has been well established in the preparation of porous 3-dimensional scaffolds for tissue engineering, while it is unsuitable for the incorporation of proteins such as growth factors and cytokines owing to the denaturation during drying at room temperature. Our group have recently developed a method to fabricate porous scaffolds of synthetic biodegradable polymers by combining porogen leaching and freeze-drying techniques using prepre-pared ice particulates as the porogen material.[85] This method might control both the pore structure of scaffolds

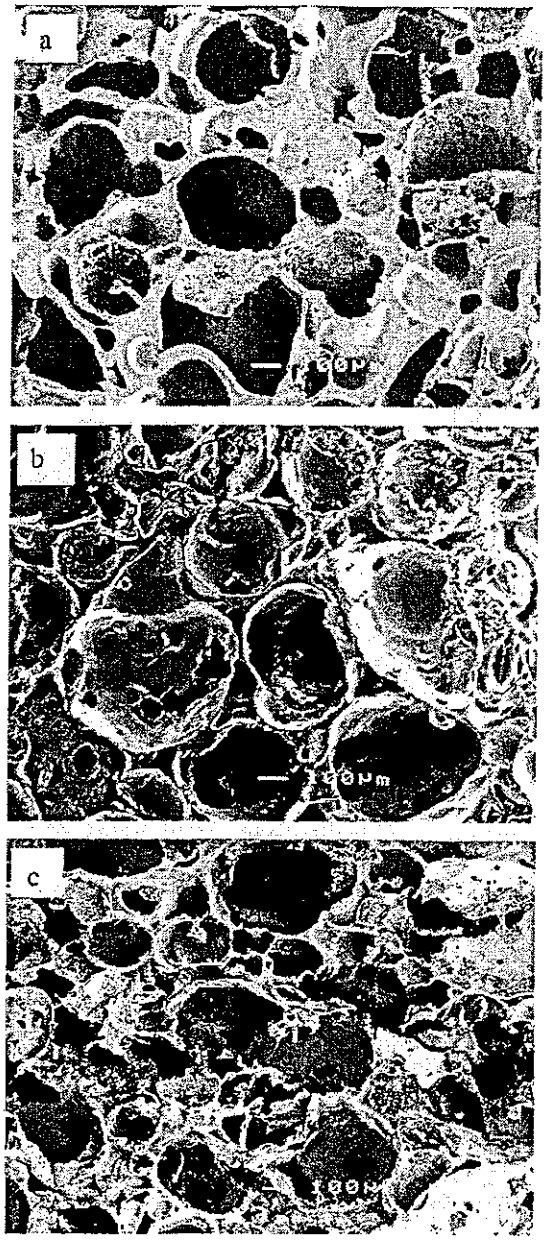

Figure 1. SEM photomicrographs of cross sections of PLLA sponges prepared with the weight fractions of ice particulates of 70% (a), 80% (b), and 90% (c).

and be used for protein incorporation. Ice particulates were formed by injecting cold deionized water into liquid nitrogen through a capillary. The formed ice particulates were almost spherical. Their diameters could be controlled by the spraying speed and travel distance. Polymer solutions in chloroform were precooled to −20 °C, then mixed with the ice particulates. The dispersion was then vortexed and poured into an aluminum mold. This process was conducted in a cool room (4 °C). The dispersion was frozen by placing the mold in liquid nitrogen, freeze-dried for 48 h under liquid-nitrogen freezing and for another 48 h at room temperature to completely remove the solvent to form the synthetic polymer sponges.

The PLLA sponges prepared by this method were highly porous with evenly distributed and interconnected pore structures (Figure 1). The pore shapes were almost the same as those of the ice particulates. The degree of interconnection within the sponges increased as the weight fraction of the ice particulates increased. The polymer concentration also had some effect on the pore wall structure, i.e., lower polymer concentration resulted in more porous pore wall structures. The porosity and surface area/weight ratio increased with the increase of the weight fraction of the ice particulates. Therefore, the pore structure of the porous temporary scaffolds could be manipulated by varying the shape, weight fraction, size of the ice particulates, and the polymer concentration.

The use of ice particulates as the porogen material enabled replacement of the conventional method of porogen leaching by repeated washing with water with freeze-drying. The porogen removal thus became easier and more complete. Also various growth factors could be incorporated into the porous polymer scaffold by choosing appropriate solvents to dissolve biodegradable polymer, which will not denature growth factors.

Hybridization of Synthetic Biodegradable Polymers with Collagen

The aforementioned two kinds of biodegradable polymers have their respective advantages and drawbacks. The synthetic biodegradable polymers are easily formed into desired shapes with relatively good mechanical strength. Their periods of degradation can also be manipulated by controlling the crystallinity, molecular weight, and copolymer ratio. Despite these advantages, the scaffolds derived from synthetic polymers lack cell-recognition signals, and their hydrophobic property hinders smooth cell seeding. In contrast, naturally derived collagen has the potential advantages of specific cell interactions and hydrophilicity, but scaffolds constructed entirely of collagen have poor mechanical strength. Therefore, these two kinds of biodegradable polymers have been hybridized to combine the advantageous properties of both constitutes.[75,77,86-91]

A collagen–PLLA hybrid scaffold with parallel collagen fibers embedded within a PLLA matrix has been prepared by dipping collagen fibers in a 10% (w/v) solution of PLLA in chloroform and drying under vacuum overnight at room temperature.[86] The tensile strength and modulus of collagen–PLA hybrids increase two-fold. Subcutaneous fibrous tissue in-growth is improved and implant resorption is slightly delayed in the collagen–PLLA hybrids. When PLLA sponges coated with collagen or infiltrated with a collagen gel are used as the 3D porous scaffold of hepatocytes, the hepatocytes show

enhanced cell survival and albumin secretion. A hybrid tube of PGA mesh-coated with collagen has been used for nerve regeneration across a 25-mm gap and shown to be a promising tool for use as a nerve guide tube in peripheral nerve regeneration. Collagen gel embedded with a PGA mesh has been used to construct epithelium similar to human esophageal epithelium.

The use of collagen gel produces a complete loss of pore structure. Compared to collagen gel, collagen sponge is porous enough to accommodate implanted cells, and its microporous structure facilitates cell seeding. Recently, a new kind of hybridization technique has been developed by introducing collagen microsponges into the pores of synthetic polymer sponges or the interstices of synthetic polymer meshes.[89–91]

To prepare the hybrid sponges of synthetic polymers and collagen, the sponges of synthetic polymers were immersed in a collagen solution under a vacuum so that the sponge pores filled with collagen solution. The synthetic polymer sponges containing the collagen solution were then frozen and freeze-dried to allow the formation of collagen microsponges in the sponge pores. The collagen microsponges were further crosslinked by treatment with glutaraldehyde. After being washed with deionized water and freeze-dried, the hybrid sponges of synthetic polymers and collagen were formed.

The hybrid structure of the PLGA–collagen hybrid sponge is shown in Figure 2. Collagen microsponges with interconnected pore structures were formed in the pores of the PLGA sponge. Scanning electron microscopy (SEM)-electron probe microanalysis of elemental nitrogen indicates that microsponges of collagen were formed in the pores of the PLGA sponge and that the pore surfaces were coated with collagen.

The wettability of a polymer scaffold is considered very important for homogeneous and sufficient cell seeding in three dimensions. Because biodegradable synthetic polymers are relatively hydrophobic, it is difficult to deliver a cell suspension in a manner that uniformly distributes transplanted cells throughout their porous scaffolds. Numerous techniques have been used to evenly seed cells in PLA- and PLGA-based porous scaffolds, such as prewetting the scaffold with ethanol solution and then replacing it with distilled water and cell suspension, introducing hydrophilic poly(ethyl glycol) into the hydrophobic PLA and PLGA network, or hydrolysis.[92–94] Hybridization with collagen improved the wettability of synthetic sponges with water, which facilitated cell seeding.

The hybrid sponges of synthetic polymers and collagen possessed almost the same high degree of mechanical strength as those of synthetic polymers, much higher than that of collagen sponges alone.

Mouse fibroblast L929 cells (Figure 2c) adhered well to the collagen microsponges of the hybrid sponge. More cells adhered to the hybrid sponges than to the sponges

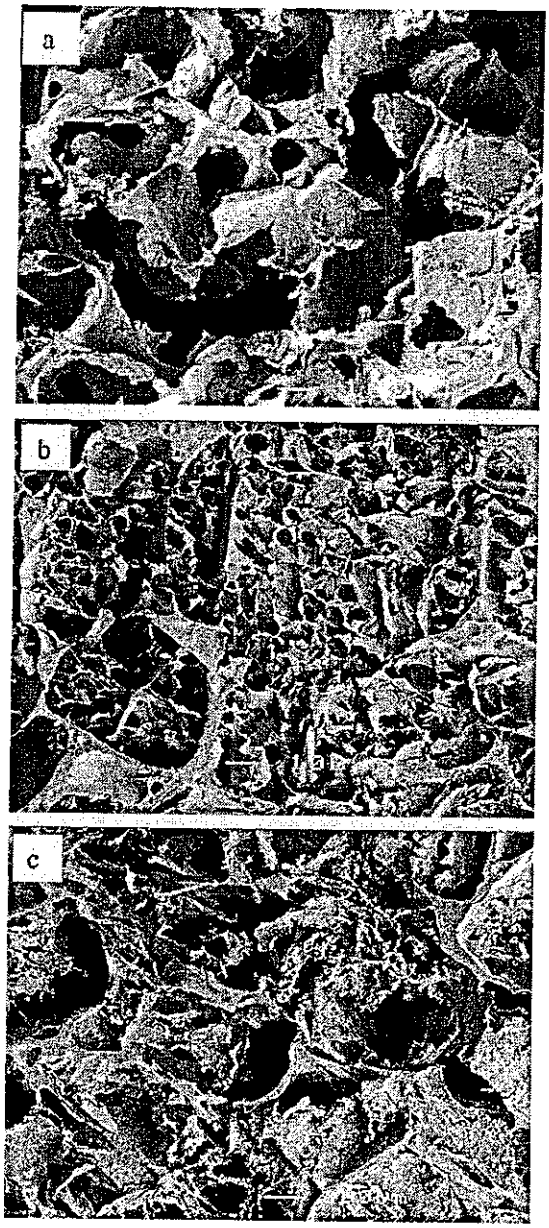

Figure 2. SEM photomicrographs of cross sections of PLGA sponge fabricated by particulate-leaching menthod (a), PLGA–collagen hybrid sponge (b), and L929 cells cultured in the hybrid sponge for one day (c).

made of synthetic polymers. The cells that adhered to the hybrid sponges proliferated and secreted their respective extracellular matrices with culture time. The hybrid sponges showed a good degree of cell interaction and biocompatibility. The sponges are prime candidates for use as porous scaffolds for the tissue engineering of cartilage, bone, liver, blood vessel, and esophagus, etc.

By the similar method, the synthetic biodegradable polymer meshes have been hybridized with collagen sponge to combine their advantages to form a new kind

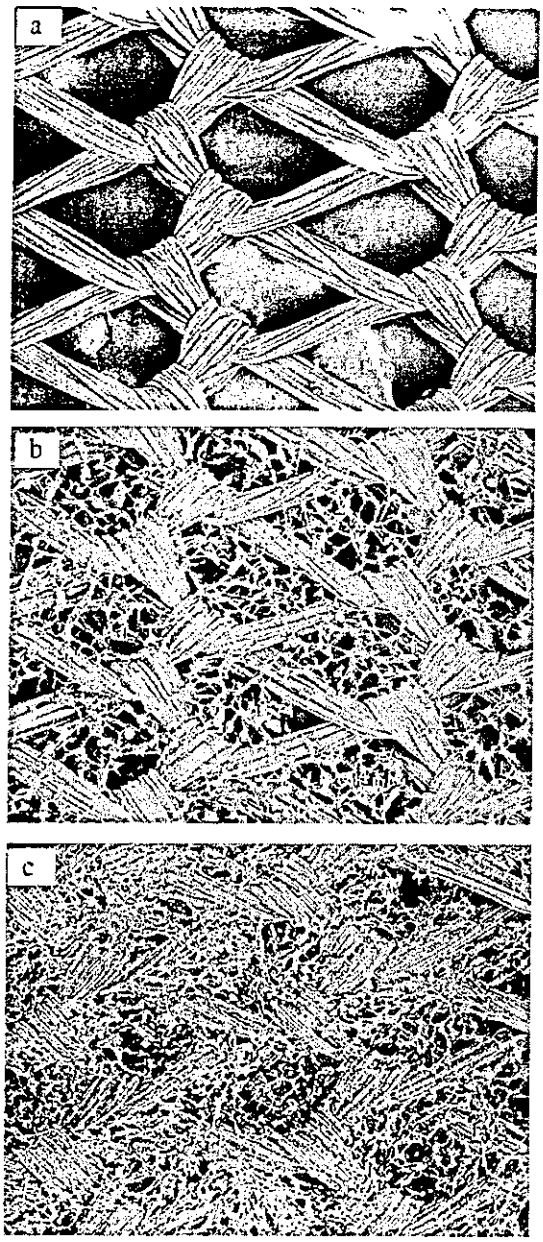

Figure 3. SEM photomicrographs of PLGA knitted mesh (a), PLGA–collagen hybrid mesh (b), and human fibroblasts cultured in the hybrid mesh for one day (c).

of hybrid mesh.[95] The hybridization was achieved by forming collagen sponges between the interstices of synthetic biodegradable polymer mesh. A hybrid mesh of Vicryl knitted mesh made of polylactin 910 and collagen is shown in Figure 3b. Collagen microsponges with interconnected microporous structures were formed in the interstices of the synthetic polymer mesh. The polymer mesh was embedded in the collagen sponge sheet so that the fiber bundles of polymer mesh and the collagen sponges were alternately chained.

The hybrid mesh possessed almost the same mechanical property as that of the polymer mesh, much higher than that of the collagen sponge alone. Human skin fibroblasts adhered and spread well on the surfaces of the collagen sponge of the hybrid mesh after being cultured for 5d (Figure 3c). After 2 weeks, they proliferated to become completely connected in a layer structure. These results suggest good cell interaction of the hybrid mesh.

A hybrid scaffold of PLLA braid and collagen sponge has also been fabricated for ligament regeneration (Figure 4).[96] Cellular responses of ligament cells to the PLLA and PLLA–collagen hybrid braids were evaluated both in vitro and in vivo. More cells and more homogeneous cell distribution were observed in vitro in the hybrid braid than in the PLLA braid. More fibroblast immigration and neo-angiogenesis were detected in the hybrid braid than in the PLLA braid when implanted on ruptured medial collateral ligament. Hybridization with collagen facilitated cell seeding and spatial cell distribution, and promoted cell immigration and neo-angigenesis.

The polymer sponge, mesh, or braid serving as a skeleton, reinforced the hybrid sponge, mesh, or braid and resulted in easy handling, while the collagen sponge provided the hybrids with a microporous structure and hydrophilicity, and therefore, easy cell seeding. These hybrid scaffolds could be useful for tissue engineering.

Hybridization of Polymers and Inorganic Hydroxyapatite

Three-dimensional scaffolds for bone tissue engineering should be osteoconductive so that osteoblasts and osteoprogenitor cells can adhere, migrate, differentiate, and synthesize new bone matrix. Hydroxyapatite has a composition and a structure very close to natural bone mineral and, therefore, has frequently been used for implants, coatings, and scaffolds in bone tissue engineering.[97–103] Hydroxyapatite particulates have been incorporated into poly(α-hydroxyl acids) to construct hybrid three-dimensional porous scaffolds of poly(α-hydroxyl acids) and hydroxyapatite. The incorporated hydroxyapatite shows a good degree of biocompatibility and osteoconductivity. In natural bone, hydroxyapatite is deposited in an orderly manner on the collagen matrix.[104] Additionally, it has been reported that the osteoconductivity of hydroxyapatite can be further improved by associating a protein matrix with the mineral structure.[105] To mimic the unique structure of collagen and hydroxyapatite in natural bone, several kinds of hybrid biomaterials of collagen and hydroxyapatite have been developed.[101,102,105–107] Because a reconstructed collagen matrix is mechanically too weak, maintaining its original shape is difficult when it is immersed in an aqueous solution, making the process of hybridization difficult to control.

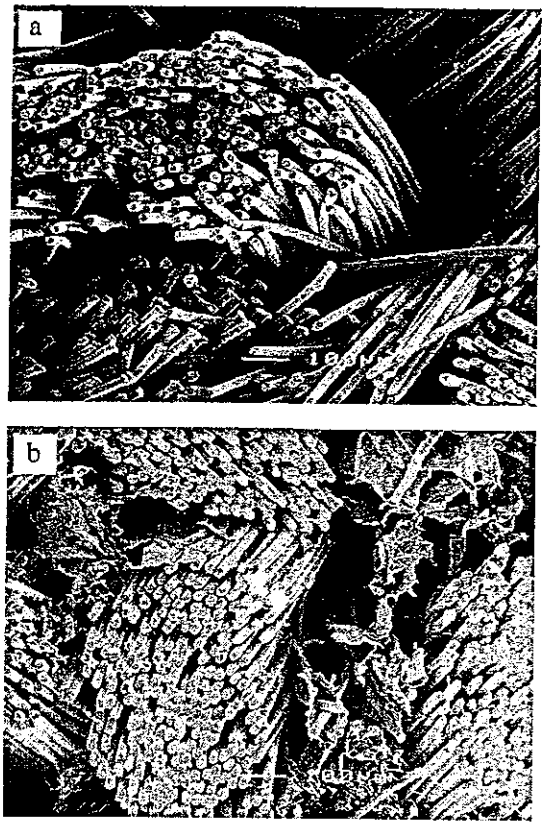

Figure 4. SEM photomicrographs of cross sections of a PLLA brade (a), and PLLA–collagen hybrid braid (b).

Recently, a hybrid sponge of synthetic polymer, collagen, and hydroxyapatite has been developed by depositing hydroxyapatite particulates on the collagen microsponge surfaces of hybrid sponge of synthetic polymer and collagen to mimic the unique structure of collagen and hydroxyapatite in natural bone.[108] The deposition of hydroxyapatite particulates was accomplished by alternate immersion of the PLGA-collagen sponge in $CaCl_2$ and Na_2HPO_4 aqueous solutions and centrifugation. The PLGA-collagen sponge was first immersed in a $CaCl_2$ solution under a vacuum to fill the sponge spaces with the solution, then incubated in the solution at 37 °C for 12 h. After removal from the $CaCl_2$ solution, the sponge was centrifuged to remove the excess $CaCl_2$ solution from the sponge. The sponge was then immersed in a Na_2HPO_4 aqueous solution to introduce the solution into the sponge after which it was then incubated in the solution at 37 °C for another 12 h. After removal from the Na_2HPO_4 solution, the sponge was again centrifuged, this time to remove the excess Na_2HPO_4 solution. This process of alternate immersion and centrifugation was defined as one cycle. Hydroxyapatite particulates were deposited on the PLGA–collagen sponge by repeating the cycles.

With the mechanically strong PLGA–collagen sponge as a template, the PLGA–collagen–hydroxyapatite hybrid sponge was able to maintain its original shape. Centrifugation after each immersion removed the excess aqueous solution remaining on the surfaces of the collagen sponges, avoiding over-precipitation of the hydroxyapatite onto the collagen surfaces. Figure 5 shows the SEM photomicrographs of PLGA–collagen–hydroxyapatite hybrid sponges after two, four, and six cycles of alternate immersion and centrifugation. Hydroxyapatite particulates were formed on the surfaces of the collagen sponges of the PLGA–collagen sponge. After one immer-

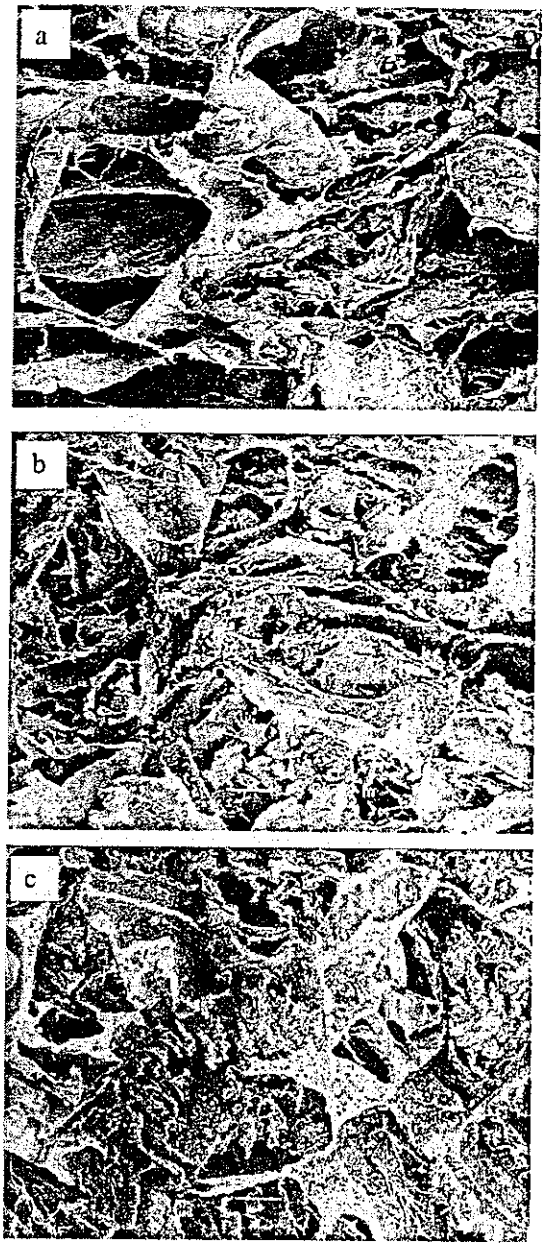

Figure 5. SEM photomicrographs of cross sections of PLGA–collagen–hydroxyapatite hybrid sponges after two (a), four (b), and six cycles (c) of alternate immersion.

sion cycle, the deposited particulates were scarce and small. They became denser and grew larger as the number of alternate immersion cycles increased. The surfaces of the collagen microsponges were completely covered with the flake-like hydroxyapatite particulates after three cycles of alternate immersion.

Energy-dispersive spectroscopy analysis showed that the calcium-to-phosphorus molar ratios in the hybrid sponge increased with the number of immersion cycles and became almost the same as that of hydroxyapatite after four cycles. X-ray diffraction (XRD) showed that the characteristic absorption peaks of hydroxyapatite were very weak in the initial immersion and became more evident after the second and third immersion cycles. After four alternate immersion cycles, the XRD spectra became almost the same as that of commercially available hydroxyapatite. These results suggest that the deposited hydroxyapatite particulates increased, grew larger, and that their level of crystallinity increased as the number of immersion cycles increased. The deposited crystals became hydroxyapatite-like after four cycles of alternate immersion.

The use of synthetic polymer sponge as a mechanical skeleton facilitated the formation of the hybrid sponges into desired shapes and gave the hybrid sponges good mechanical properties. Because collagen and hydroxyapatite are the two primary components of extracellular bone matrix, they should provide the hybrid sponge with a good degree of cell interaction and osteoconductivity. Such synthetic polymer–collagen–hydroxyapatite hybrid sponges would prove useful as 3D porous scaffolds for bone tissue engineering.

Conclusion and Outlook

Many biodegradable biomaterials have been used and fabricated into various shapes for tissue engineering. These scaffolds showed promising results, guiding tissue development. The processing of the chosen materials into appropriate three-dimensional scaffolds with desired shapes and pore structures will be critical. Although the porous three-dimensional scaffolds possess interconnected highly porous structures, development of scaffolds allowing easy cell seeding and spatially even distribution of transplanted cells remains to be challenged. The concept of combining synthetic polymers with naturally derived polymers and hydroxyapatite is very attractive and seems effective. The skeleton of synthetic polymers defines the gross shape and size of the engineered tissue and supports the forming tissue during the initial stages, while the embedded collagen sponge or collagen–hydroxyapatite sponge facilitates cell invasion and growth. Hybrid scaffolds possess the favorable properties of synthetic polymers, naturally derived polymers and hydroxyapatite. As the progenitor cells for different tissue have isolated, the control of differentiation in these cells would be another challenge for scaffold design.

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