A Cell Leakproof PLGA-Collagen Hybrid Scaffold for Cartilage Tissue Engineering

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A cell leakproof porous poly(ε-lactic-co-glycolic acid) (PLGA)-collagen hybrid scaffold was prepared by wrapping the surfaces of a collagen sponge except the top surface for cell seeding with a bi-layered PLGA mesh. The PLGA-collagen hybrid scaffold had a structure consisting of a central collagen sponge formed inside a bi-layered PLGA mesh cup. The hybrid scaffold showed high mechanical strength. The cell seeding efficiency was 90.0% when human mesenchymal stem cells (MSCs) were seeded in the hybrid scaffold. The central collagen sponge provided enough space for cell loading and supported cell adhesion, while the bi-layered PLGA mesh cup protected against cell leakage and provided high mechanical strength for the collagen sponge to maintain its shape during cell culture. The MSCs in the hybrid scaffolds showed round cell morphology after 4 weeks culture in chondrogenic induction medium. Immunostaining demonstrated that type II collagen and cartilaginous proteoglycan were detected in the extracellular matrices. Gene expression analyses by real-time PCR showed that the genes encoding type II collagen, aggrecan, and SOX9 were upregulated. These results indicated that the MSCs differentiated and formed cartilage-like tissue when being cultured in the cell leakproof PLGA-collagen hybrid scaffold. The cell leakproof PLGA-collagen hybrid scaffolds should be useful for applications in cartilage tissue engineering. © 2009 American Institute of Chemical Engineers Biotechnol. Prog., 26: 819–826, 2010

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Introduction

Tissue engineering that combines three-dimensional porous scaffolds and cells has been widely studied as an attractive approach in the treatment of malfunctioning or lost tissues and organs.1,2 Stem cells or committed cells are seeded in a porous scaffold and cultured in vitro or in vivo to form a new tissue or organ for transplantation. Biodegradable porous scaffolds play an important role in tissue engineering to accommodate cells, control cell adhesion and proliferation, and guide new tissue formation. Naturally derived polymers such as collagen3,4 and biodegradable synthetic polymers, such as poly(glycolic acid) (PGA),5,6 poly(ε-lactic acid) (PLA),7 poly(L-lactic acid) (PLA),8 poly(ε-lactic-co-glycolic acid) (PLGA),6,8 and poly(ε-caprolactone),9 have been used to construct three-dimensional porous scaffolds for tissue engineering. However, collagen and the biodegradable synthetic polymers have their respective advantages and drawbacks.10 Collagen contains the Arg-Gly-Asp (RGD) motif for cell adhesion and its hydrophilic property is beneficial for cell penetration and diffusion in its porous scaffolds. However, collagen-derived porous scaffolds have very poor mechanical strength. They deform and lose their original shapes during cell culture. In contrast, porous scaffolds made from biodegradable synthetic polymers have high mechanical strength. However, synthetic polymer scaffolds have no cell adhesion motif and their surfaces are hydrophobic, which results in requiring pretreatment before use for cell culture. Hybridization of collagen and biodegradable polymers has been proposed to combine their advantages and overcome their weaknesses. A hybridization

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method has recently been developed by forming collagen sponge or collagen microsponges in a mechanical skeleton of synthetic polymers. Several kinds of such hybrid porous scaffolds have been reported. The hybrid porous scaffolds combined the advantages of collagen and synthetic polymers, showing easy cell seeding, good cell adhesion, and high mechanical properties. These hybrid scaffolds have been used for tissue engineering of bone, cartilage, ligament, skin, cardiovascular, etc.

Except for the pore structure and good mechanical properties, protection against cell leakage during cell seeding is required to retain more cells in the porous scaffold to facilitate efficient tissue regeneration. Hybrid porous scaffolds do combine the advantages of their components. However, porous scaffolds have a common problem of cell leakage during cell seeding because of their highly porous structure. If cells escape from the porous scaffold during cell seeding, there might be a significant loss of seeded cells, therefore adversely affecting the efficiency of tissue regeneration. This is important because the cell source from a patient is limited. The requirement of efficient use of a patient’s cells drives the development of new methods to solve the leakage problem. Although many methods have been developed to facilitate cell seeding by controlling pore structure, surface modification, and hybridization, the development of new strategies to protect against cell leakage remains a challenge. In this study, a novel PLGA-collagen hybrid scaffold was developed by forming collagen sponge in a PLGA mesh cup. All the surfaces of the collagen sponge except the top surface were covered with a PLGA mesh. The PLGA mesh cup had a bi-layered structure: a PLGA knitted mesh having big interstices and a PLGA woven mesh having small interstices. The PLGA knitted mesh was used to prevent the central collagen sponge from shrinking while the PLGA woven mesh protected against cell leakage. The cell leakage-proof PLGA-collagen scaffold was used for the culture of human bone marrow-derived mesenchymal stem cells (MSCs) for cartilage tissue engineering.

Materials and Methods

Preparation of hybrid scaffold

The preparation scheme of the cell leakage-proof PLGA-collagen hybrid scaffold is shown in Figure 1a. First, a PLGA bi-layered membrane was prepared by gluing a PLGA knitted mesh (VICRYL™; Johnson & Johnson, New Brunswick, NJ) with a PLGA woven mesh (VICRYL™). A solution of PLGA was used as the glue. The PLGA solution was prepared by dissolving 1.0 g PLGA (molar ratio of lactic to glycolic acid in the copolymer = 75:25, Mn 66,000 – 107,000 Da; Sigma-Aldrich, St. Louis, MO) in 5 mL chloroform. The PLGA solution glue was spread on one side of a PLGA woven mesh to coat it. A PLGA knitted mesh was put on the coated surface of the woven mesh and pressed to glue the two meshes together. Then, a rectangular disc of the PLGA bi-layered mesh was formed into a tube by gluing the short sides of the rectangular mesh disc with the knitted mesh inside and woven mesh outside. Finally, the bi-layered PLGA tube was glued to a square of the bi-layered PLGA scaffold.
mesh to form a mesh cup with the PLGA knitted mesh inside and the PLGA woven mesh outside. The inner diameter of the cup was 6 mm and its height was 5 mm.

Collagen sponge was introduced into the PLGA mesh cup to prepare the cell leakproof PLGA-collagen hybrid scaffold. The mesh cups were immersed in a bovine collagen type I acidic solution (0.5%, pH 3.2, Koken, Tokyo, Japan) and vacuumed to completely fill the interstices of the mesh cups with the collagen solution. The PLGA mesh cups were then put in a silicone mold that just fit the PLGA mesh cup. The silicone mold was used to prevent the collagen aqueous solution from flowing out during the next process. After the PLGA mesh cup was set in the silicone, the 0.5% collagen aqueous solution was poured into the central space of the PLGA mesh cup. The mesh cup containing the collagen solution was frozen at −80°C for 4 h, and the ice that formed above the top surface of the mesh cup was trimmed after freezing. The frozen collagen/mesh cup constructs were freeze-dried under a vacuum of 25 Pa for 24 h to form a collagen sponge. The collagen sponge was cross-linked by treatment with glutaraldehyde vapor from a saturated 25% glutaraldehyde aqueous solution at 37°C for 4 h. After the cross-linking, the sponge was treated with a 0.1 M glycine aqueous solution to block unreacted aldehyde groups. After being completely washed with water and freeze-dried, a cell leakproof PLGA-collagen hybrid scaffold was obtained. Each scaffold was sterilized with 70% ethanol aqueous solution and used for cell culture.

**Mechanical testing**

The PLGA-collagen hybrid scaffold and collagen sponge prepared using the same process but without the PLGA mesh cup were subjected to a static compression test. The collagen sponge was used as a control. The mechanical properties of the scaffolds were measured by a static compression test machine (TMI UTM-10T; Toyo Baldwin, Tokyo, Japan). Each test sample was compressed at a rate of 1.0 mm/min, in dry condition at room temperature (RT). The elastic modulus and stiffness were determined from the load-deformation curve and the dimensions of each sample using the following two equations. The average elastic modulus and stiffness were calculated from six samples.

\[
E = \frac{F}{A_0} \left/ \left( \frac{\Delta L}{L_0} \right) \right.
\]

\[
S = \frac{F}{\Delta L},
\]

where \( E \) is Young’s modulus (modulus of elasticity, MPa); \( S \) is stiffness (N/mm); \( F \) is the force applied to the object; \( A_0 \) is the original cross-sectional area through which the force is applied; \( \Delta L \) is the deformation; and \( L_0 \) is the original length of the object.

**Cell culture**

Mesenchymal stem cells derived from human bone marrow were obtained from Osiris (Worthington Biochemical, Lakewood, NJ) at passage 2. The MSCs were subcultured twice in T-75 culture flasks using the proliferation medium from Osiris and used for culture in the scaffolds at passage 4. The proliferation medium contained 440 mL MSC basal medium, 50 mL mesenchymal cell growth supplement, 10 mL 200 mM l-glutamine, and 0.5 mL penicillin/streptomycin mixture. The cells were collected by treatment with trypsin/ethylenediaminetetraacetic acid (EDTA) solution and suspended in the proliferation medium at a density of 1.5 × 10⁶ cells/mL. The PLGA-collagen hybrid scaffolds and collagen sponges were placed in the wells of six-well cell culture plates. The collagen sponges were used as a control. Each of the scaffolds was seeded with 1 mL cell suspension solution. After culture for 30 min, the scaffolds were moved to new six-well culture plates. The cells in each well of the old plates were collected. These were cells that leaked from the scaffolds during cell seeding. The number of leaked cells was counted using a hemacytometer. The difference between the number of seeded cells and the number of leaked cells was considered to be the number of cells that adhered in each sponge. The seeding efficiency was calculated by dividing the number of adhered cells by the number of seeded cells. Five samples were used for these measurements to obtain averages and standard deviations.

The seeded MSCs were cultured in the proliferation medium for 1 week. After culture for 1 week in the proliferation medium, the culture medium was changed to a chondrogenic induction medium. The cells were cultured in the chondrogenic induction medium for another 4 weeks. The chondrogenic induction medium consisted of serum-free Dulbecco’s modified Eagle medium (DMEM) containing 4,500 mg/L glucose, 584 mg/L glutamine, 100 U/mL penicillin, 10 μg/mL streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 50 mg/L ascorbic acid, 10⁻⁷ M dexamethasone, and 10 ng/mL TGF-β3 (Sigma-Aldrich). The medium was changed every 3 days.

**SEM observation**

The porous structure of the bi-layered PLGA mesh cup, the PLGA-collagen hybrid scaffold and the cell distribution in the PLGA-collagen hybrid scaffolds were observed by a scanning electron microscope (SEM) (JSM-6400Fs; JEOL, Tokyo, Japan). The PLGA-collagen hybrid scaffold was sectioned with a razorblade and the cross-sections were coated with platinum using a sputter coater (Sanyu Denshi, Tokyo, Japan). For SEM observation of cultured cells, three cell-seeded PLGA-collagen hybrid scaffolds were removed from the culture plate 3 h after cell seeding, washed three times with phosphate buffered saline (PBS), and fixed with 0.5% glutaraldehyde solution in PBS at RT for 4 h. After being rinsed three times each with PBS and deionized water, the cell-seeded scaffolds were freeze-dried, cross-sectioned, and coated with platinum for SEM observation.

**Histological and immunohistological staining**

After chondrogenic induction culture in the PLGA-collagen hybrid scaffold for 4 weeks, the MSCs were harvested and fixed in neutral buffered formalin. The samples were embedded in paraffin and sectioned (8-μm thick). The cross-sections were stained with hematoxylin and cosin. The type I collagen, type II collagen, and cartilage proteoglycan were immunohistologically stained using rabbit anti-human type I collagen antibody (Sanbio BV, Uden, The Netherlands), mouse anti-human type II collagen monoclonal antibody (NeoMarkers, Fremont, CA), mouse anti-human cartilage proteoglycan monoclonal antibody (Chemicon International, Temecula, CA), and a DAKO-Universal LSAB Kit, Peroxidase (Dako, Carpinteria, CA) according to the instructions accompanying the kit. Briefly, the deparaffinized sections were incubated with proteinase K enzyme in Tris-buffered
saline (1:50 working dilution, pH 7.4) at RT for 5 min, and blocked with peroxidase blocking solution for 10 min and 10% goat serum solution for 30 min. The sections were then incubated with anti-type I collagen (1:400 working dilution), anti-type II collagen (1:200 working dilution), and anti-proteoglycan antibodies (1:1200 working dilution) for 1 h. Incubation with biotinylated anti-rabbit or mouse immunoglobulins for 30 min was followed by incubation with horseradish peroxidase-conjugated streptavidin for 10 min. The sections were then incubated with 3-amino-9-ethylcarbazole as a color substrate for 10 min to visualize the bound antibodies. The nuclei were counterstained with hematoxylin. All incubations were conducted at RT.

RNA isolation and real-time PCR

The MSCs cultured in the PLGA-collagen hybrid scaffold in the chondrogenic induction medium for 4 weeks were washed with PBS. The samples were frozen in liquid nitrogen. The frozen samples were crushed into powder by an electric crusher. The powder from each sample was dissolved in 1 mL Isogen reagent (Nippon Gene, Toyama, Japan) and the RNA was isolated. DNase-treated RNA was treated with RQ1 RNase-free DNase (Promega) prior to being converted to cDNA by AMV Reverse Transcription (Takara Bio). Total RNA (1 µg) was reversely transcribed into cDNA using random hexamer (Applied Biosystems) in 20 µL reaction. Real-time PCR was amplified for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), types I, II, and X collagen, sox9, and aggrecan. The reaction was performed with 1 µL cDNA, 300 and 150 nM each PCR primer and PCR probe, and 12.5 µL TaqMan Universal PCR Master Mix (Applied Biosystems). Reactions were cycled using a Bio-Rad iCycler for 40 cycles. After an initial incubation step of 2 min at 50°C and denaturation for 10 min at 95°C, 40 cycles (95°C for 15 s, 60°C for 1 min) of PCR were performed. Reactions were performed in triplicate. 18S levels were used as endogenous controls and gene expression levels relative to GAPDH recombinant RNA were calculated using the comparative Ct method. Three samples under each condition were used for measurement to calculate the mean values and standard deviations. The primer and probe sequences (Applied Biosystems) followed those of Martin et al.19 and Schaefer et al.20. These sequences were:

Figure 2. SEM photomicrographs of (a) the outer PLGA woven mesh and (b) inner PLGA knitted mesh of the bi-layered PLGA mesh cup, and (c) the horizontal cross-section of the central area, (d) vertical cross-section of the central area, (e) cross-section of the bottom area and (f) cross-section of the peripheral area of the PLGA-collagen hybrid scaffold. A–C indicates the central collagen sponge, PLGA knitted mesh and PLGA woven mesh, respectively.
and that of the inner PLGA knitted mesh ranged from 250 to 200 $\mu$m. The pore size of the collagen sponge ranged from 30 to 10 $\mu$m. Therefore, the inner PLGA knitted mesh was necessary to prevent the shrinking of the collagen sponge.

The intertwined structure between the large openings of the inner PLGA knitted mesh and collagen sponge fibers prevented detachment of the collagen sponge and maintained the integrity of the scaffold.

**Statistical analysis**

For statistical analyses, the data were analyzed by ANOVA using the SYSTAT 11.0 software package. If treatments were determined to be significant, pairwise comparisons were performed using Bonferroni’s adjustment. Differences were considered significant for $P < 0.05$.

**Results**

**Microstructure and mechanical property of the PLGA-collagen hybrid scaffold**

A cell leakproof PLGA-collagen hybrid scaffold was prepared by introducing collagen sponge in a bi-layered PLGA mesh cup that had an inner PLGA knitted mesh and an outer PLGA woven mesh. The inner PLGA knitted PLGA mesh and outer PLGA woven mesh were bound together by a PLGA collagen solution in chloroform. To investigate the necessity of the bi-layered structure, a single-layer cup of the PLGA-collagen hybrid scaffold were 0.36 $\pm$ 0.09 MPa and 0.07 $\pm$ 0.02 N/mm, respectively. However, those of the collagen sponge were 0.03 $\pm$ 0.06 MPa and 0.07 $\pm$ 0.02 N/mm, respectively. The elastic modulus and stiffness of the PLGA-collagen hybrid scaffold after MSCs were cultured for 3 h were about 12 and 18 times greater than were those of the collagen sponge. The PLGA mesh cup served as a mechanical skeleton to reinforce the central collagen sponge.

**Cell seeding efficiency and cell distribution**

Human bone marrow-derived MSCs were cultured in the PLGA-collagen hybrid mesh. Cell seeding efficiency was measured with the collagen sponge as a control. The cell culture medium in the cell suspension solution passed through the PLGA mesh of the scaffold. The cell seeding efficiencies of the PLGA-collagen hybrid scaffold and collagen sponge were 90.0 $\pm$ 2.7% ($n = 5$) and 23.1 $\pm$ 1.2% ($n = 5$), respectively. The cell leakproof PLGA-collagen hybrid scaffold showed significantly higher cell seeding efficiency than did that of the collagen sponge alone ($p < 0.05$). These results indicate that the bi-layered PLGA mesh cup
reduced cell leakage during cell seeding and, therefore, increased cell seeding efficiency. The outer PLGA woven mesh of the cup had small interstices and might help prevent cell leakage from the scaffold.

SEM observation of horizontal and vertical cross-sections of the hybrid scaffold after the MSCs were cultured in the hybrid scaffold for 3 h demonstrated that the MSCs adhered and distributed homogenously in the scaffold (Figure 3).

**Histological staining and gene expression**

Histological examination of the cells cultured in the PLGA-collagen hybrid scaffold for 4 weeks in the chondrogenic induction medium supplemented with TGF-β3 using hematoxylin and eosin stains indicated cells with a round morphology (Figure 4a). Immunohistological staining with antibodies to type I collagen, type II collagen, and cartilage proteoglycan showed that the type I collagen, type II collagen, and cartilage proteoglycan were positively stained in the MSC culture in the PLGA-collagen hybrid scaffold (Figures 4b–d).

The gene expression of type I collagen, type II collagen, type X collagen, sox9, and aggrecan was examined by real-time PCR (Figure 5). The MSCs cultured in the PLGA-collagen hybrid scaffold expressed a significantly higher level of these genes than did the passage 4 MSCs. All these genes were significantly upregulated when the MSCs were cultured in the hybrid scaffold. The results of gene expression coincided with the histological and immunohistological results, which suggest the chondrogenic differentiation of the MSCs.

**Discussion**

Collagen sponge scaffolds have been used for the tissue engineering of various tissues and organs such as bone, cartilage, tooth, skin, tendon, ligament, etc.21–23 The collagen sponges provide good cell adhesion and cell proliferation, and promote tissue regeneration. However, collagen-derived porous scaffolds are mechanically weak. They are usually deformed as cells shrink during cell culture. It is difficult to maintain the new tissues in their initially designed shapes because the collagen scaffolds are too weak to withstand cell contraction during tissue regeneration. The low mechanical strength of these scaffolds also makes medical manipulation difficult. To solve these problems, collagens have been hybridized with mechanically strong materials into hybrid structures. One such major set of scaffolds is the hybrid scaffolds of collagen with biodegradable synthetic polymers such as polyesters and polycaprolactones. Chen et al. have reported a hybridization method by forming collagen microsponges in the openings of a synthetic polymer skeleton.11,12 The synthetic polymer skeleton facilitates formation into desired shapes and provides the appropriate mechanical strength, while the collagen microsponges facilitate cell seeding and cell attachment. Tabata et al. have reported a collagen sponge reinforced by the incorporation of PGA fiber.24,25

In this study, we hybridized collagen sponge with a PLGA mesh cup to form a hybrid structure with collagen sponge formed in the center of a bi-layered PLGA mesh cup. PLGA was used because it has gained the approval of the US Food and Drug Administration for certain human clinical use, such as surgical sutures and some implantable devices. The
hybrid structure not only reinforced the collagen sponge giving the same effect as reported before, it also effectively prevented cell leakage from the porous structure during cell seeding. The bi-layered mesh structure of the cup was central to maintain the integrity of the scaffold and prevent cell leakage. The inner PLGA knitted mesh with large interstices intertwined with the collagen sponge fibers, and therefore held the collagen sponge in close contact and prevented collagen sponge shrinkage. The outer PLGA woven mesh had small interstices and therefore prevented cell leakage from the porous structure. The pores of outer PLGA woven mesh were around a few \( \mu \text{m} \), which were permeable to liquid, nutrient and gas but not to cells. With the cell leakproof effect of the PLGA-collagen hybrid scaffold, cells could be seeded at a low concentration when the same amount of cells is seeded in a porous scaffold. Usually high cell concentration has been used to seed cells in conventional porous scaffolds because a low concentration of cells requires that more solution be loaded, resulting in a loss of solution together with cells. However, use of a high seeding concentration cell suspension solution might result in cell aggregation and negatively affect the distribution of cells in the scaffold. A low seeding concentration is preferable for cell delivery and homogenous cell distribution. The PLGA-collagen hybrid scaffold in the present study could prevent cell leakage, enabling the seeding of cells at a low concentration to improve cell distribution throughout the scaffold.

Human bone marrow-derived MSCs showed a homogenous distribution in the PLGA-collagen hybrid scaffold when they were cultured in the hybrid scaffold. The cells showed a round morphology and expressed genes encoding cartilaginous extracellular matrices. They secreted cartilaginous extracellular matrix (ECM) such as type II collagen and aggrecan. Cartilage-like tissue was regenerated after the MSCs were cultured in the hybrid scaffold for 4 weeks. Type I collagen was also detected in the ECM by immunostaining. This might be mostly synthesized during the early stage of culture because the MSCs secreted type I collagen before chondrogenic induction. However, the expression level of the type I collagen gene was significantly suppressed compared to the great increase of type II collagen gene. A longer period of culture will further result in a decrease of type I collagen.

The surrounding bi-layered PLGA mesh structure will also provide some advantages for in vivo implantation. The PLGA mesh can be sutured with surrounding host tissue to help in fixation of the implant when the hybrid scaffold is used for in vivo application for the treatment of articulator...
cartilage. The cell and secreted ECM will not only fill the space in the hybrid scaffold, but also replace the space when the scaffold is degraded. The newly regenerated tissue will be integrated with surrounding host tissue.

Conclusions

A new hybrid porous scaffold of collagen and PLGA was prepared by forming collagen sponge in a bi-layered PLGA mesh cup. The PLGA-collagen hybrid scaffold prevented cell leakage, increased cell seeding efficiency, and showed high mechanical strength as compared with collagen sponge not in a mold. The bi-layered PLGA mesh cup provided the hybrid scaffold with high mechanical properties and prevented cell leakage while the collagen sponge facilitated cell adhesion and cell distribution. Cartilage-like tissue was regenerated when MSCs were cultured in the hybrid scaffold. The cell leakproof PLGA-collagen hybrid scaffold will be a useful scaffold candidate for cartilage tissue engineering applications.

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Literature Cited


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