The effects of pore size in bilayered poly(lactide-co-glycolide) scaffolds on restoring osteochondral defects in rabbits

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Abstract: Bilayered porous scaffolds have recently attracted interest because of their considerable promise for repairing osteochondral defects. However, determination of optimal pore size in bilayered porous scaffolds remains an important issue. This study investigated the in vivo effects of pore size in bilayered scaffolds using a rabbit model of osteochondral defects. We fabricated five types of integrated bilayered poly(lactide-co-glycolide) (PLGA) scaffolds with different pore sizes in the chondral and osseous layers (50–100 μm, 100–200 μm, 200–300 μm, and 300–450 μm). A subset of bilayered scaffolds seeded with or without allogenic bone marrow mesenchymal stem cells (BMSCs) was implanted in rabbit osteochondral defects. All of the cell/scaffold composite constructs supported the simultaneous regeneration of articular cartilage and subchondral bone, but the best results were observed in cell-seeded PLGA scaffolds with 100–200 μm pores in the chondral layer and 300–450 μm pores in the osseous layer. Our study supports the concept that the effects of pore size on osteochondral repair should be taken into consideration during scaffold design for tissue engineering.

Key Words: osteochondral defect, bilayered scaffold, poly(lactide-co-glycolide), stem cell, tissue engineering


INTRODUCTION

Repairing articular cartilage lesions remains one of the most challenging problems in the orthopedic field. A variety of strategies have been utilized in clinical practice to solve this issue, including microfracture, debridement, and implantation of tissue grafts or cells.3–4 The results have been unsatisfactory; at long-term follow-up, the repaired tissues are found to be composed of fibrocartilage rather than hyaline cartilage. As an alternative to current therapies that have fallen short, the scaffold-based method has been applied into repairing the damaged cartilage.5–8 However, treatments that focus exclusively on articular cartilage defects are likely to fail9 because mature articular cartilage lacks blood vessels and innervation and damaged cartilage has difficulty obtaining sufficient nutrition.1 Therefore, it has been suggested that treatment strategies should be designed with the entire osteochondral unit (articular cartilage and subchondral bone).5 Because a bone-to-bone interface integrates better and faster than a cartilage-to-cartilage or cartilage-to-bone interface,10 the anomalous cartilage can obtain the nutrition from the subchondral bone, and then be repaired successfully. Most scaffolds have a single layer; but a homogenous biomaterial is not ideal for supporting the metabolic and morphogenic activities of chondrocytes and osteoblasts.11 which need different microenvironments and have different requirements.5,12

Therefore, some investigators are designing bilayered porous scaffolds that better mimic the different physiological properties and structures of both articular cartilage and subchondral bone.6,13–20 Bilayered scaffolds in the form of osteochondral plugs have been developed in an attempt to simultaneously regenerate articular cartilage and subchondral bone layers, and encouraging studies using these materials have been reported.6,13–20 These studies reported different bilayered scaffolds with pore diameters ranging from 5 to 800 μm.13,14,17–19,21,22

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TABLE I. Integrated Bilayered PLGA Scaffolds Prepared in this Study*

<table>
<thead>
<tr>
<th>Bilayered PLGA Scaffold</th>
<th>Chondral Layer</th>
<th>Osseous Layer</th>
</tr>
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<tbody>
<tr>
<td>Scaffold A</td>
<td>50–100</td>
<td>300–450</td>
</tr>
<tr>
<td>Scaffold B</td>
<td>100–200</td>
<td>300–450</td>
</tr>
<tr>
<td>Scaffold C</td>
<td>200–300</td>
<td>200–300</td>
</tr>
<tr>
<td>Scaffold D</td>
<td>300–450</td>
<td>100–200</td>
</tr>
<tr>
<td>Scaffold E</td>
<td>300–450</td>
<td>50–100</td>
</tr>
</tbody>
</table>

*All scaffolds were 85% in porosity, 4 mm in diameter, and 5 mm in thickness (chondral layer: 1 mm; osseous layer: 4 mm).

Some employed smaller pores in the chondral layer and larger pores in the osseous layer, whereas others used the same sized pores throughout the scaffold. Although pore size plays a significant role in biological delivery and tissue regeneration, it is unknown whether different pore sizes in the two layers of a bilayered scaffold affect osteochondral defect repair. Further investigation is required to optimize pore sizes in bilayered scaffolds. However, to the best of our knowledge, no specific study has examined the effect of pore size on the in vivo efficacy of repairing osteochondral defects with bilayered scaffolds, although some reviews summarize the existing literature. Therefore, it is necessary to assess the effects of different pore sizes in the two layers of bilayered scaffolds on the in vivo osteochondral repair process.

Here, we fabricated five types of integrated bilayered poly(lactide-co-glycolide) (PLGA) scaffolds with different pore sizes in the chondral and osseous layers. PLGA is a widely used biodegradable material with excellent biocompatibility, biodegradability, and mechanical strength. PLGA scaffolds are suitable for cartilage repair, partly due to their appropriate degradation in vivo, which may reduce macrophage infiltration. The attenuation of inflammatory cytokines and subsequent tissue reactions results in high-quality regenerated cartilage. Importantly, PLGA is one of a few synthetic materials that have been approved by the United States Food and Drug Administration for clinical applications.

We seeded the scaffolds with allogenic bone marrow-derived mesenchymal stem cells (BMSCs), which are considered a promising tissue regeneration source. They are easily harvested and do not cause donor site morbidity or elicit an immune response. They are also capable of differentiating into osteoblasts, chondrocytes, and other cell types when placed in a suitable niche. We implanted bilayered scaffolds with or without BMSCs in the chondral layer into osteochondral defects in rabbit knee joints and assessed the effects of pore size on osteochondral repair in vivo.

MATERIALS AND METHODS
Ethics statement
This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Fudan University (permit number: 2008-0039). All surgeries were performed under the anesthesia with ketamine hydrochloride, and all efforts were made to minimize suffering.

Preparation of integrated bilayered PLGA scaffolds
PLGA with a lactic/glycolic (LA/GA) molar ratio of 85/15 (PLGA85/15) was purchased from Purac (Netherlands), and the inherent viscosity was 2.36 dL/g. PLGA porous scaffolds were fabricated by a “room-temperature” compression molding/particulate leaching method as described previously. Dichloromethane was employed as the solvent to dissolve the PLGA, and sodium chloride (NaCl) particulates were used as porogen. The mixture of the PLGA solution and porogen was pressed into a predesigned mold and kept under pressure. A cylindrical form (4 mm in diameter, 10 mm in height) was obtained after releasing the mold. Two mixtures with porogens of different pore sizes were glued together with a small amount of dichloromethane. After cutting the mixture constructs into appropriate sizes, porogen was leaching by water, and the bilayered scaffolds were eventually obtained. Five types of integrated bilayered porous scaffolds with identical porosity (85%) and different pore sizes (50–100 μm, 100–200 μm, 200–300 μm, and 300–450 μm) in the two layers were fabricated in this article. The scaffold characteristics are listed in Table I.

The cylinder scaffolds were 4 mm in diameter and 5-mm thick [chondral layer: 1 mm, osseous layer: 4 mm; Fig. 1(B)]. Scaffold microstructure was observed with scanning electron microscopy (SEM). The scaffolds were sterilized with ethylene oxide prior to implantation.
BMSC culture

We employed our previously described protocol for identifying rabbit BMSCs. Three-month-old New Zealand white rabbits were selected for BMSCs isolation and culture. Bone marrow aspirates (5 mL) were obtained from rabbit iliac crests and subsequently cultured as our previous methods. Briefly, isolated cell pellets were resuspended in 5-mL complete medium comprised of Dulbecco’s modified Eagle’s medium with low glucose (DMEM-LG, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco, Australia), 100 mg/mL streptomycin, and 100 U/mL penicillin. The cells were seeded in T-25 flasks (Corning) and cultured in a 37°C and 5% CO₂ incubator. Nonadherent cells were removed when the medium was changed after 24 h. After that, the medium was replaced every 3 days until the cells reached 80% confluence. Then, the cells were washed twice with phosphate-buffered saline (PBS), detached by treatment with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA, Sigma), and subcultured at 1:3 under the same conditions until the third passage.

Cell labeling

To track BMSCs, cells were stained with a fluorescent lipophilic tracer, 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, Invitrogen) according to the manufacturers’ protocol. Third passage BMSCs were trypsinized and resuspended at 1 × 10⁶ cells/mL in DMEM-LG, and DiI was added at 5 μL/mL. After incubation in the dark at 37°C with 5% humidified CO₂ for 20 min and two washes with DMEM-LG, DiI-labeled cells were resuspended in complete medium for use. DiI is a membrane-bound fluorescent dye with very low cell toxicity that does not compromise cell viability or differentiation potential. Even if the dye was to leach out of dead cells, it would not emit significant fluorescence in an aqueous environment like the cartilage matrix. Prior to use, the samples were observed under an inverted fluorescence microscope (Olympus IX51). The labeling efficiency was checked by flow cytometry (BD FACS Aria II), using BMSCs without DiI as control.

BMSC seeding into bilayered scaffolds

The sterilized scaffolds were placed in 24-well culture plates (Corning) and incubated in DMEM-LG overnight to improve cell seeding efficiency. BMSCs in complete medium at a concentration of 5 × 10⁷ cells/mL were divided into 20-μL aliquots that were evenly seeded drop by drop into the chondral layer of bilayered scaffolds using a 1-cc syringe. The cell-seeded scaffolds (1 × 10⁶ cells/scaffold) were incubated under standard conditions for 2 h to allow cells to adhere to the scaffold prior to the addition of 1 mL fresh complete medium, then incubated for 7 days with medium changes every 3 days. The cell-free scaffolds were incubated in the same conditions as controls.

SEM examination

After being cultured for 7 days, the cell-seeded scaffolds were taken out of the culture well and fixed in 2.5% glutaraldehyde at 4°C for 24 h. Thereafter, the samples were sequentially dehydrated in 30, 50, 70, 90, and 100% ethanol, critical-point dried, sputter-coated with gold, and examined with SEM (TS5136MM, TESCAN).

Surgical implantation

Thirty-six skeletally mature New Zealand white rabbits (5–6 months old) weighing 3.0–3.3 kg were used in the study. The rabbits were anesthetized with ketamine hydrochloride (35 mg/kg) after a 1-week acclimation. The knee regions were shaved and disinfected, and medial parapatellar incisions were made on the bilateral knee joints to expose the femoral condyles. Osteochondral defects (4 mm in diameter and 5 mm in depth) centered on the femoral condyles were created with a surgical drill bit. Penetration depth was limited by a line marked on the drill bit. All debris was removed from the defect with a curette, and the defect was flushed with sterile normal saline. Thereafter, the cell-seeded scaffold and the corresponding cell-free scaffold were press-fitted into the medial and lateral condyles in each knee, respectively.

The cell-seeded and cell-free scaffolds were considered as one implant pair. Each type of five different scaffolds had 12 pairs, which were randomly assigned to 12 knees in 36
rabbis. Autologous osteochondral plug were implanted into the medial condyle as a positive control, and the lateral condyle defect in the same knee remained blank as a negative control. The experimental groups and time points are listed in Table II.

After implantation, the knee joint synovia, capsule, and skin were closed layer by layer. The rabbits were fed tap water and food ad libitum, kept in separate cages, and allowed to move freely. Gentamycin (4 mg/kg) was injected intramuscularly once a day for 3 days after surgery.

**Tissue retrieval and histological analysis**

At 6 and 12 weeks postoperatively, rabbits were sacrificed by ketamine hydrochloride injections. The samples were harvested, examined, and photographed. Then, some 6-week samples were cut in half; one half was quickly frozen in liquid nitrogen, embedded in optimum cutting temperature (OCT) (Tissue-Tek, CA 90501) compound, and cryosectioned at 10-μm intervals with a freezing microtome at −20°C (Leica CM1850, Germany). Cryosections were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) and observed under an upright fluorescence microscope (Olympus BX51) to visualize immunoreactivity.

The 12-week samples from scaffolds with or without cells (n = 6/group). One rabbit (four osteochondral defects) died from diarrhea 4 weeks after implantation of scaffold D and scaffold E. Histological analysis included hematoxylin and eosin, toluidine blue, safranin O/fast green staining, and immunohistochemical assessment of collagen types I and II.

**Real-time PCR**

Samples were homogenized with a Tissue-Tearor. Total RNA was isolated using TRizol reagent (Invitrogen) according to the manufacturer’s protocol. The cDNA was reverse-transcribed with a Reverse Transcription System (TaKaRa). Real-time PCR was performed using Brilliant SYBR Green QPCR master mix (TaKaRa) in an ABI 9700 real-time PCR system under the conditions of 15 s at 95°C, 1 min at 60°C, and the fluorescence intensity was recorded for 40 cycles. Each real-time PCR run was performed with at least three experimental replicates, and the expression level of target gene was normalized against that of glyceraldehyde 3-

**TABLE II. Experimental Groups and Time Points**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of Samples for Histological Analysis</th>
<th>Number of Samples for PCR</th>
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<tbody>
<tr>
<td>Scaffold A</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Scaffold B</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Scaffold C</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Scaffold D</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Scaffold E</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Blank control group</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Autologous osteochondral plug</td>
<td>6</td>
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*Thirty-six rabbits (144 osteochondral defects) were randomly assigned to either a control group or one of five types of scaffolds with or without cells (n = 12/group). One rabbit (four osteochondral defects) died from diarrhea 4 weeks after implantation of scaffold D and scaffold E. Histological analysis included hematoxylin and eosin, toluidine blue, safranin O/fast green staining, and immunohistochemical assessment of collagen types I and II.*
phosphate dehydrogenase (GAPDH). PCR primer sequences are provided in Table III.

Statistical analysis
All data are expressed as mean ± standard deviation (SD). The histological grading and gene expression among different types of scaffolds were analyzed with one-way analysis of variance (ANOVA), and statistical analysis of grading between the same scaffolds was performed using Student’s t-tests. The significance level was set as p < 0.05.

RESULTS
Characterization of bilayered scaffolds
The PLGA scaffolds were generated by a porogen leaching method using NaCl particulates ranging from 50 to 450 μm, and five types of bilayered scaffolds were obtained by combining two scaffold layers with the pore sizes summarized in Table I. The pore structure of each layer is shown in Figure 4, and the pores were interconnected with each other. The interface of the bilayered scaffold consists of two firmly integrated layers with different pore sizes. The weight ratio of particulate and polymer were the same in all five scaffold types, and the porosity was approximately 85% as assessed with an improved liquid replacement method.23 Because the chondral and osseous scaffold layers were of different pore sizes and tightly combined, they are technically integrated bilayered scaffolds.

Observation of seeded BMSCs in vitro and tracking implanted BMSCs in vivo
SEM indicated that BMSCs spread well on the scaffold pore walls (Fig. 5). We also tried to track implanted BMSCs. The cells were prelabeled with Dil prior to implantation (~97% labeling efficiency, as determined by flow cytometry; Fig. 2(C)]. Red fluorescence in the neotissues was visible under an upright fluorescence microscope 6 weeks after implantation. Many cells were labeled by both Dil and DAPI (Fig. 6), suggesting that implanted BMSCs in the defects were viable and able to participate in restoring osteochondral defects.

Macroscopic specimen observation
All rabbits tolerated the bilateral operations well and were ambulatory immediately following recovery from anesthesia. The gross examination of knee joint did not show inflammation of the synovial membrane or other joint tissues.

![Figure 4](image-url) SEM images of the five types of integrated bilayered scaffolds. The images (A–E) show the pore structures of scaffolds A–E, respectively. The pore sizes are listed in Table I. The yellow dashed lines indicate the boundaries between the layers. (F–J) Corresponding magnified images of (A–E).
In the blank control group, the defect was partially filled with amorphous soft fibrous tissue at 6 weeks postoperation. After 12 weeks, the obvious vacancy in the center of defect could still be seen in the lateral condyle [Fig. 7(F)], indicating poor self-repair ability as reported in previous studies. Most defects in those groups of cell-free scaffolds were covered with an irregular tissue 6 weeks after implantation. All the defects were filled with varying degrees of regenerated tissues after 12 weeks, and they were better than those observed in the blank control. Reparative tissues in the cell-seeded bilayered scaffolds exhibited better resurfacing than those in the corresponding cell-free scaffolds (Fig. 7).

Gross and cross-sectioned observations revealed that the defects repaired with cell-seeded scaffold B (chondral layer: 100–200 μm; osseous layer: 300–450 μm) were entirely filled with a smooth cartilage-like tissue similar to the adjacent normal cartilage. The new tissue was well integrated without visible demarcation. The tissues repaired by other implants were not as smooth as those by cell-seeded

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**FIGURE 5.** SEM images of sections of PLGA scaffolds loaded with BMSCs after 1 week of culture. A: Cells were uniformly distributed throughout the chondral layer of scaffold D and adhered tightly. B: Higher magnification of the orange rectangle in (A), with yellow arrows indicating BMSCs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**FIGURE 6.** Fluorescent images of neotissues 6 weeks after implantation. The images (A–E) show fluorescent images of tissues repaired by implantation of cell-seeded scaffold A–E, respectively. Dil-labeled cells were clearly observed in the DAPI-stained neotissue, with yellow arrows indicating Dil-labeled cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
scaffold B (Figs. 7 and 8), and the tissue repaired by scaffold E even remained an obvious border with the surrounding tissue.

**Histological examination**

In the blank control group, H&E staining showed just a small amount of new trabecular bone in the subchondral region and an irregular surface layer of fibrous tissue even after 12 weeks. In contrast, 6 weeks after implantation, PLGA materials could be observed in all scaffold types and were padded with fibrous or undifferentiated cells. The defects implanted by scaffolds with cells were filled with irregular fibrous tissue mixed with immature cartilage in the chondral layer and small bony islands in the subchondral region. Although the defects implanted with cell-free scaffolds showed inferior repair in the chondral layer, the 6-week histological grading scores were significantly different between scaffolds with and without cells (Fig. 9), but not among the different types of scaffolds.

After 12 weeks, most sections showed decreased residual scaffold material in the lower portion of subchondral

![Images of reparative tissues 12 weeks after implantation](image-url)

**FIGURE 7.** Macroscopic appearances of reparative tissues 12 weeks after implantation. Images (A–E) show the defects repaired by scaffold A–E (with or without cells), respectively. The defects in the medial (M) and lateral (L) condyles were repaired with a cell-seeded scaffold and a cell-free scaffold, respectively. Image (F) indicates the auto-osteochondral plug and control treatment in the medial and lateral condyles, respectively. Of five types of scaffolds, neotissues in the medial condyles exhibited better defect resurfacing than those in the lateral condyles, as indicated by the red dotted rings in the defect sites. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

![Images of cross-sectional views of reparative tissues 12 weeks after implantation](image-url)

**FIGURE 8.** Cross-sectional view of reparative tissues 12 weeks after implantation. Images (A–E) show the cross-sectional views of the neotissues by implantation of cell-seeded scaffolds (A–E), respectively. Images (F and G) display those of neotissues by auto-osteochondral plug and control treatments, respectively. The red arrows indicate the interfaces between the neotissues and native tissues. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
bone, suggesting appropriate degradation of our PLGA scaffolds. Residual scaffold E was most obvious [Fig. 10(A1–E1)]. All of the 12-week reparative tissues had a higher percentage of hyaline cartilage and better bone regeneration [Figs. 10 and 11] than those at 6 weeks. Cell-seeded scaffold A had a higher level of reparative tissues integrate with the native tissue; type II collagen was expressed in the chondral region, and newly formed bone with robust type I collagen expression was well integrated with native bone [Fig. 11(A1–A4)]. Cell-seeded scaffold B showed that the neotissue was integrated completely with native tissue without obvious borders.

There was a visible tidemark between cartilage layer and subchondral bone layer; but the newly formed cartilage layer was thicker than the native cartilage [Fig. 10(B1)]. Toluidine blue and safranin O/fast green staining revealed that the reparative tissues had extensive dark staining with hyaline-like chondrocytes parallel to the surface in the superficial zone and orderly aligned in the deep layer [Fig. 10(B3 and B4)]. Immunohistochemical experiments demonstrated stronger expression of collagen type II in the articular surface and robust expression of collagen type I in the subchondral region [Fig. 11(B1–B4)]. In defects repaired with cell-seeded scaffold C, the reparative tissue was also mostly integrated with surrounding tissue. An appropriate amount of collagen I and II could be detected in the neotissues, but the tidemark was absent. Defects implanted with cell-seeded scaffold D also contained neotissue with moderate amounts of collagen II and collagen I that was mostly integrated with host tissue. The lowest level of collagen II expression was found for cell-seeded scaffold E; most of the scaffold material was still in the osseous layer.

The 12-week grading scores illustrated significant differences not only between the scaffolds with and without cells, but also among the different types of scaffolds with cells (Fig. 9). The mean total score for cell-seeded scaffold B was the lowest, indicating that such a combination of pore sizes in our bilayered PLGA scaffolds facilitated the best tissue repair.

Gene expression analysis
We used real-time PCR to quantify gene expression in neotissues repaired by different cell-seeded scaffolds after 12 weeks (Fig. 12). In cell-seeded scaffold B, the relative level of collagen type II was significantly higher than those from other experimental groups ($p < 0.05$); although the mean level of aggrecan was higher in cell-seeded scaffold B defects, the difference was not statistically significant compared to other groups. As for the relative level of collagen type I, we observed a significant difference between
scaffolds B and D ($p < 0.05$). Notably, some samples expressed a very high level of collagen type I, which lead to larger variations of the measured values. And the relative levels of the three genes we assessed in each group were still lower than the levels of the autologous osteochondral group.

**DISCUSSION**

Repairing osteochondral lesions involving both cartilage and underlying bone remains a serious challenge in regenerative medicine. Driven by increasingly serious medical needs, there have been many investigations of osteochondral repair. In recent years, scaffold-based regenerative medicine has attracted considerable interest, including the application of bilayered scaffolds that mimic the physiological properties of two different tissues. In this study, we fabricated bilayered yet integrated porous PLGA scaffolds, which were seeded with allogenic BMSCs to simultaneously regenerate cartilage and subchondral bone. This design is based on the recognition of the different requirements to repair cartilage and bone in osteochondral defects, as well as the need to prevent delamination of the different components. The results confirmed that delamination did not occur at all.

Besides the design of bilayered scaffolds, scaffold pore size is a crucial variable in repairing osteochondral

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**FIGURE 10.** Histological images of tissues repaired by cell-seeded scaffolds 12 weeks after implantation. Images (A1–E1, magnifications in A2–E2) show the results of H&E staining for tissues repaired by cell-seeded scaffolds A–E, respectively. (A3–E3) and (A4–E4) depict toluidine blue and safranin O/fast green staining, respectively, and the regions are similar to those depicted in (A2–E2). It can be seen that (B1–B4) exhibit the best tissue morphology.
defects. As far as surface cartilage regeneration is concerned, pore sizes less than 200 μm are thought to promote chondrogenesis, which requires a hypoxic environment to create a sparsely vascularized tissue with low mechanical properties. However, pore sizes should not be less than 50–100 μm to facilitate cell seeding and migrating. With regards to osteogenesis, the pore diameter is usually between 200 and 600 μm. Pore sizes larger than 300 μm are recommended for promoting vascularization and new bone formation. Oversized pores are prone to diminished mechanical properties and compromise tissue regeneration. Moreover, it was reported that polycaprolactone scaffolds with pores between 350 and 800 μm exhibited little pore size effect on bone regeneration. Although the pore size effect on osteogenesis in the single layer scaffold has been examined, the effects of different pore sizes in bilayered scaffolds had not yet been specifically examined, to the best of our knowledge.

Our present study is aimed at finding optimal pore size distribution in bilayered PLGA scaffolds to facilitate osteochondral repair. We designed five types of integrated bilayered scaffolds. Figure 11 shows immunohistochemical images of tissues repaired by cell-seeded scaffolds 12 weeks after implantation. Images (A1–E1) show collagen type II staining in tissues repaired with cell-seeded scaffolds A–E, respectively. (A3–E3) depict collagen type I reactivity. (A2–E2) and (A4–E4) are high-magnification images of the rectangles in (A1–E1) and (A3–E3), respectively. Defects repaired with cell-seeded scaffold B had the best restorative results (B1–B4). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
scaffold layer thickness and are not a good match for the necessary mechanical properties of scaffolds. So, the examined pore sizes are among 50–450 μm.

In our study, osteochondral defects were created on the medial and lateral condyles of the main weight-bearing zones of the knee, and then bilayered scaffolds seeded with BMSCs and corresponding cell-free scaffolds were implanted (Fig. 3). All cell-seeded scaffolds repaired the defects, yet to different extents. We also found that the neotissues generated with cell-seeded scaffold B had the best gross appearance [medial condyle of Fig. 7(B)] and cross-sectional view [Fig. 8(B)] after 12 weeks. The subsequent histological analysis (Figs. 10 and 11) and real-time PCR results (Fig. 12) further supported the superiority of scaffold B. These results suggest that 100–200 μm pores and 300–450 μm pores promote chondrogenesis and osteogenesis/vascularization, respectively.

This type of paired structure in bilayered scaffolds is in accord with other reports that described smaller pores in the chondral layer and larger pores in the osseous layer.14,17,21 There were several other differences between their studies and ours: they did not set control groups with various pore sizes in their in vivo tests, and they employed a composite biomaterial rather than PLGA. For these reasons, the data from those studies cannot be directly compared, and our observations are not contradictory with previously published reports.

BMSCs have garnered interest in fundamental research and even in clinical trials.18,27,30,34,41,42 Here, we used allogenic BMSCs to repair osteochondral defects and did not observe obvious immune reactions or synovial inflammation. It is known that allogenic stem cells lack significant immunogenicity, which permits their allogenic transplantation without immunosuppressive drugs.43 To monitor the fate of allogenic BMSCs, Dil was used to label BMSCs prior to implantation. A previous study demonstrated that Dil remains fluorescent for at least 24 weeks after implantation.25 Similarly, we clearly observed fluorescent cells in neotissues after 6 weeks (Fig. 6). Although the number of Dil-labeled cells decreased obviously at 12 weeks, sparse fluorescent cells could be seen (data not shown). The findings suggest that allogenic BMSCs facilitate tissue repair.

We did not add any induction medium in the present study. Nevertheless, BMSCs have been known to differentiate in vitro on material surfaces, even without the addition of soluble induction media.44,45 Implantation of composite constructs of BMSCs and PLGA porous scaffolds into sheep illustrated unambiguously that the local in vivo microenvironment could trigger BMSCs differentiation into tissue cells matching those microenvironments.46 It was also reported that BMSCs are more likely to undergo chondrogenesis with the architectural support of three-dimensional PLGA scaffolds.47 Therefore, it is reasonable that undifferentiated BMSCs could, after being seeded in both layers of scaffolds, spontaneously differentiate into chondrocytes or osteoblasts in vivo without external induction media.

In addition, even without preseeding of allogenic BMSCs in the osseous layer of bilayered scaffolds, new cancellous bone formed in the subchondral region and integrated with the host tissue, which is in agreement with previous reports.17,19 It is tempting to speculate that endogenous cells penetrate into the scaffolds and participate in tissue regeneration, similar to the “cell homing” concept advocated in recent publications.6,42,43

It should be mentioned that there are still several limitations in our study. From the scaffold perspective, the chondral layer is thicker than native cartilage in rabbit knee joints. Because the follow-up period after implantation in vivo was only 12 weeks, the long-term outcomes for the regenerated tissue such as mechanical properties are unclear. Further optimization of bilayered scaffolds and additional experimental studies in larger animals are necessary to more closely mimic conditions of human synovial joints.

CONCLUSIONS
This study examined the effects of pore size in integrated bilayered PLGA scaffolds on regeneration in articular osteochondral defects in rabbits. The integrated bilayered scaffolds seeded with allogenic BMSCs in the chondral layer supported the simultaneous regeneration of both articular cartilage and subchondral bone, and we found that pore sizes of 100–200 μm in the chondral layer and 300–450 μm in the osseous layer generated the best results. Although optimal pore size values cannot simply be applied to other
scaffolds, our findings underscore the importance of this scaffold design parameter for tissue engineering and regenerative medicine.

REFERENCES