Bilayered PLGA/PLGA-HAp Composite Scaffold for Osteochondral Tissue Engineering and Tissue Regeneration

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Supporting Information

ABSTRACT: This study is aimed at investigation of the osteochondral regeneration potential of bilayered PLGA/PLGA-HAp composite scaffolds with one layer made of biodegradable polymer poly(D,L-lactide-co-glycolide) (PLGA) and another layer made of PLGA polymeric matrix coated by bioactive ceramics hydroxyapatite (HAp). The composite scaffolds were fabricated by compression molding/particle leaching and plasma-treated surface deposition. The pore morphology, mechanical properties, and surface deposition of the scaffold were characterized, and the growth of bone marrow derived mesenchymal stem cells or medicinal signaling cells (MSCs) in the scaffold was verified. Thereafter, rabbit models with an artificial osteochondral defect in joint were randomized into three treatment groups: virgin bilayered scaffold, bilayered scaffold preseeded in vitro with MSCs, and untreated blank control. At 16-week postoperation, both the virgin scaffolds and cell-seeded bilayered scaffolds exhibited osteochondral repair, as verified by biomechanics analysis, histological evaluations, and Western blot. The results highlighted the potentiality of the bilayered PLGA/PLGA-HAp composite scaffold for osteochondral tissue engineering, and in particular tissue regeneration or in situ tissue induction, probably by recruiting the local cells toward chondrogenic and osteogenic differentiation in the porous biomaterials.

KEYWORDS: bilayered scaffold, biodegradable polymers, composite materials, tissue regeneration, osteochondral repair

INTRODUCTION

Articular cartilage lesions are a common and increasingly serious problem due to disease, sports, and progressive aging, and the lesions can cause persistent symptoms of joint pain and constrained motion of the knee joints. Once damaged, articular cartilage has minimal self-healing due to no blood supply and limited chondrocyte migration constrained by the rich extracellular matrix (ECM) in this tissue. The repair of an articular cartilage defect has thus been an intractable challenge in the field of regenerative medicine.

In order to treat the articular cartilage lesions, researchers have developed arthroscopic microfracture, arthroplasty, osteotomy, autologous chondrocyte implantation, and conservative treatments. These techniques are limited by defect size, availability of medical care, or induction of donor-site morbidity. Tissue engineering emerges as a promising alternative strategy for cartilage regeneration by implanting porous scaffolds to provide a three-dimensional support for cell growth and in situ tissue regeneration. However, repair of cartilage merely by a single-layer scaffold probably brings about the problem of poor integration between cartilage and subchondral bone. It has been reported that articular cartilage cannot be independently repaired without assistance from subchondral bone. The subchondral bone plays, as a strong support of cartilage, an important role on stress transmission in the knee joints. Moreover, articular cartilage and subchondral bone are two tissues with different moduli, components, physiological structures, and physical functions, indicating that

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a single-layer scaffold cannot fully meet the requirements of osteochondral repair. Hence, the rehabilitation of the cartilage layer or chondral layer might be carried out simultaneously with the reconstruction of the subchondral bone layer or bony layer.

Some bilayered scaffolds have been proposed to mimic the cartilage layer and subchondral bone layer. For instance, Yin’s group reported a bilayered poly(l-glutamic acid)/chitosan scaffold seeded with adipose stem cells, which was implanted into rabbit models to regenerate the hyaline-like cartilage demonstrated by the histological examination, biomechanical, and biochemical analysis. TruFit (Smith & Nephew, USA) is made of semiporous poly(D,L-lactide-co-glycolide) (PLGA), calcium sulfate, and polyglycolide (PGA) fibers, yet it has shown a controversial effectiveness for osteochondral regeneration; Agili-C (CartiHeal Ltd., Israel) is a crystalline coral aragonite/hyaluronic acid bilayer scaffold, showing the restoration of the osteochondral defect.

A bilayered scaffold seeded with cells before implanting into osteochondral defect has a positive efficacy of repair, because cells seeded on the scaffolds can be triggered to differentiate by the local in vivo microenvironment. A critical question is then raised about the necessity of cell seeding for an osteochondral bilayered scaffold, or whether a virgin bilayered scaffold alone could have an equiponderant osteochondral regeneration or not. Cell-loaded scaffolds have significant restrictions on long-time preparation, complicated examination, and expensive regulatory approval of applications. The food and drug administration (FDA) might consider a bilayered scaffold as a “medical device”, while a scaffold-cell mixture, so far, can only be regarded as a biological technique instead of a product. These regulatory requirements thereby enhance much interest in cell-free bilayered scaffolds for osteochondral repair. Therefore, it is necessary to make it clear whether or not a merely virgin scaffold can be used for an in situ osteochondral induction or regeneration.

An appropriate composite scaffold might lead to a very good repair efficacy even without introduction of any seed cells, as schematically shown in Figure 1a. The present report is aimed to fabricate a bilayered composite scaffold to check such a hypothesis as an acellular strategy.

Our group has prepared bilayered scaffolds composed of merely PLGA polymers. According to our previous studies, the scaffold with a porosity of 92% in the cartilage layer and a porosity of 77% in the subchondral bone layer exhibited the best efficacy in tissue engineering. Furthermore, pore sizes of 100–200 μm in the cartilage layer and 300–450 μm in the subchondral bone layer generated the best results. These findings of bilayered scaffold design parameters are very helpful for subsequent research for osteochondral tissue engineering and regenerative medicine.

In light of biomimetics, the merely polymeric scaffold might not be ideal for the subchondral bone. Our previous study of tissue engineering of segmental bone indicated that introduc-

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**Figure 1.** Repair of an osteochondral defect in a rabbit knee joint by a PLGA/PLGA-HAp bilayered scaffold. (a) Schematic presentation of the efficacy. “Blank control” refers to the group with the defect untreated, “Scaffold” indicates the group of implanting just a porous scaffold to examine the effect of in situ tissue regeneration, and “Scaffold with MSC” indicates the group of implanting both a porous scaffold and external seed cells. The dashed line presents the efficacy of the normal tissue. (b) Schematic presentation of the site of the defect and implant. (c) Matrix material and the fabrication of the composite bilayered porous scaffold. The cartilage layer scaffold (PLGA) and subchondral layer scaffold (PLGA/HAp) were prepared separately and then glued by dichloromethane (CH2Cl2) to obtain a bilayered scaffold.
tion of some bioceramics such as hydroxyapatite (HAp) was helpful for promoting osteogenesis. In this work, we fabricated bilayered PLGA/PLGA-HAp scaffolds and implanted them into the femoral condyles of rabbits (Figure 1b) to evaluate the physicochemical properties and biological performance of the composite scaffolds. The bilayered scaffold was fabricated by gluing the cartilage layer scaffold (PLGA) and subchondral layer scaffold (PLGA/HAp) as shown in Figure 1c.

Aliphatic polyester is a class of popular biodegradable polymers, among which PLGA is very important as a matrix for a tissue engineering scaffold because of its high adjustability of degradation rate. Sodium chloride particles are employed as porogen to fabricate the cartilage layer and subchondral bone layer of PLGA porous scaffolds by room-temperature compression molding and porogen leaching. We also examined the effect of tissue engineering by implanting porous scaffolds and seed cells. Mesenchymal stem cells or medicinal signaling cells (MSCs) derived from bone marrow were employed as a model cell type, which has been an important type of seed cells in tissue engineering. It is well-known that HAp is one of the most important elements of bone and plays an outstanding role on biomechanics and osteogenic differentiation of MSCs. In addition, HAp would buffer the acidic products of PLGA degradation and may thereby contribute to avoid an unfavorable environment for the cells and tissue owing to a decreased pH. So, we deposited the HAp on the interior surfaces by immersing them into modified simulated body fluids (SBF) so as to enhance the biocompatibility and mechanical property of the subchondral bone layer scaffolds.

We implanted as-fabricated bilayered scaffolds alone and also the scaffolds with MSCs into osteochondral defects in rabbit knee joints (Figure 1b) to assess their safety and effectiveness of some bioceramics such as hydroxyapatite (HAp) was helpful for promoting osteogenesis. In this work, we fabricated bilayered PLGA/PLGA-HAp scaffolds and implanted them into the femoral condyles of rabbits (Figure 1b) to evaluate the physicochemical properties and biological performance of the composite scaffolds. The bilayered scaffold was fabricated by gluing the cartilage layer scaffold (PLGA) and subchondral layer scaffold (PLGA/HAp) as shown in Figure 1c.

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a flow rate of 40 mL/min. We examined the weight from 50 to 800 °C at a heating rate of 20 °C/min.

**FESEM-EDS Analysis of As-Fabricated Bilayered Scaffolds.** Field-emission scanning electron microscopy (FESEM, Zeiss, Ultra 55, Germany) was carried out to observe the morphology of interior pores of bilayered PLGA/PLGA-HAp scaffold slices. Additionally, energy-dispersive X-ray spectroscopy (EDS, Zeiss, Germany) was employed to analyze the calcium–phosphorous ratio of the deposition on the interior surfaces of the subchondral-layer PLGA scaffold. Before the FESEM-EDS testing, a thin layer of gold was sputtered on the sample surface for 60 s. The heater voltage in the microscope was set at 20 kV.

**XRD Analysis of the Deposition on the Interior Surfaces of Subchondral-Layer PLGA Scaffold.** X-ray diffraction (XRD, XPert Pro, PANalytical B.V., The Netherlands) was employed to qualitatively characterize the deposition on the interior surfaces of subchondral-layer PLGA scaffold. We used commercial hydroxyapatite as standard.

**Compression Tests To Determine Mechanical Properties of the Bilayered Scaffolds at Both Dry and Wet States.** The compressive properties of the cartilage-layer, subchondral-layer, and bilayered scaffolds were characterized by measuring the stress–strain curves with the approach described in our previous work.26,27,61 Cylindrical porous scaffolds with a diameter of 4 mm and a height of 5 mm were used as testing specimens at both “dry” and “wet” states. The “dry-state” testing was conducted in a “dry” state at room temperature. For the “wet-state” tests, the specimens were prewetted with ethanol first and then with phosphate buffered saline (PBS) solution to replace the ethanol via a cyclic vacuumization and air-charge method60,62 to make sure that the specimens were fully permeated with PBS solution until completely submerged into the PBS medium. After 24-h immersion into PBS, the mechanical testing of the specimens at both “dry” and “wet” states were carried out by using an electromechanical universal testing system (5966, Instron, Norwood, MA, USA) at a speed of 0.6 mm/min until 80% strain or fracture under a constant temperature and humidity condition. After the mechanical testing, normalization processing was used to compare the moduli of “dry-state” and “wet-state” scaffolds. First, the mean value of the each group of “dry-state” modulus was regarded as each reference value (100%). Then all the relative values of the raw “dry-state” and “wet-state” moduli of each group at different scaffolds were gathered as a set, respectively. Eventually, two sets of “dry-state” and “wet-state” moduli were compared.

**MSC Seeding onto Bilayered PLGA Scaffold and In Vitro Culture.** According to the previous protocol,63–66 MSCs were isolated from the bone marrow of 7-day old neonatal Sprague–Dawley (SD) rats purchased from Shanghai Laboratory Animal Research Center (China). Before cell seeding, each scaffold slice of 4 mm in diameter and 2 mm in height were sterilized by soaking into 75% ethanol for 24 h, washed in phosphate-buffered saline (PBS, pH 7.2–7.6, Gibco) three times and then in Dulbecco’s modified Eagle medium (DMEM, Hyclone, Logan, UT, USA) for another three times.

As cultured MSCs in complete medium at a concentration of 5 × 10³ cells per mL were injected into the chondral layer of the bilayered scaffold by using a 1-cc syringe as showed in Figure 1c. The scaffolds with seeding cells were placed into 24-well culture plates (Costar, Corning, U.S.A.) and incubated at 37 °C supplemented with 5% CO₂ and 95% humidity for 2 h to make cells adhere well to the scaffold, and incubated for 7 days with the medium exchanged every 48 h before being used in the next experiment. The untreated scaffolds were incubated under the same condition, which served as the control.

**Observation of MSC Growth in Porous Scaffolds.** We observed the MSCs adhering on the interior surface of scaffolds with an SEM microscopy (VEGA 3 XMU, TESCAN, Czech). After a 7-day culture, the cell-seeded scaffolds were gently fixed with 4% paraformaldehyde at room temperature for 1 h. Then dehydration of gradient ethanol was carried out. Gold was sputtered onto the surfaces of samples for 60 s, and adherent MSCs were observed by SEM. We also used an optical microscope to observe cells on porous scaffolds after 7-day culture. Prior to the observations, filamentous actins (F-actins) and nuclei of MSCs on the scaffolds were fluorescently stained by phalloidin-tetramethyl rhodamine B isothiocyanate (phalloidin-TRITC) and 4',6-diamidino-2-phenylindole (DAPI). Briefly, MSCs on the scaffolds were thoroughly rinsed with PBS, fixed into 4% paraformaldehyde at room temperature for 10 min and rinsed with PBS for 5 min three times, then treated by 0.1% Triton X-100 for 10 min for membrane permeation and rinsed with PBS for 5 min three times again. First, in order to stain F-actins, MSCs were incubated with 1 mg/mL phalloidin-TRITC (Sigma) at room temperature in dark for 1 h, and then rinsed with PBS for 5 min three times. Afterward, the nuclei of MSCs were stained. We added 5 mg/mL DAPI (Sigma) at room temperature in dark for 10 min, and the sample was thoroughly rinsed with Milli-Q water for several times.

An inverted fluorescence microscope (Axiovert 200, Zeiss, Germany) was employed to observe the immunofluorescence of stained MSCs. The fluorescence micrographs were captured by a digital CCD camera (AxioCam HRC, Zeiss, Germany).

**Surgical Implantation.** Thirty-six skeletal mature New Zealand white rabbits (5–6 months old, randomly selective gender) with weight of 2.9–3.3 kg were enrolled in the study. After a one-week acclimation, the rabbits were anaesthetized with pentobarbital sodium (1 mL/kg), and the limbs were fixed. The knee joints were disinfected after being shaved. Then a medial parapatellar incision was made on the bilateral knee joints until the femoral condyle was exposed. A surgical drill bit (customized) with scale marks was employed to create an osteochondral defect (4 mm in diameter and 5 mm in depth) centered on the femoral medial condyle, and the defect was flushed with sterile normal saline. Thereafter, the cell-seeded and virgin bilayered PLGA/PLGA-HAp scaffolds were press-fitted into the medial condyle in each knee joint.

The cell-seeded and virgin bilayered PLGA/PLGA-HAp scaffolds were set up as one implanted pair. Rabbit knee joints were assigned randomly to the implants of two pairs in 24 rabbits (8 and 16 weeks, n = 6 for each scaffold group). Besides, the untreated lateral condyle as a normal group, and the medial condyle defect in the same knee joints remained blank as a blank control group.

After implantation, the surgical incision like joint capsule and skin were sutured layer by layer. All rabbits were fed in cages with tap water and food ad libitum and allowed to move freely. Lastly, gentamycin (4 mg/kg) was injected intramuscularly for 3 days after surgery implantation and once a day to avoid postoperative infection.

**Micro-CT Observations of the Osteochondral Defects and Efficacies of Repair of Rabbit Knee Joints.** A high-resolution micro-CT imaging system (Bruker SkyScan 1176, U.S.A.) was employed to observe the osteochondral defects and assess the repair efficacy of rabbit knee joints. The scanning parameters were set as follows: spatial resolution of 9 µm pixel size; X-ray tube voltage at 65 kV; X-ray tube current of 380 mA; optical filter of Al 1 mm; scanning angular scope of 360°, and rotation step of 0.5°.

The software GPUReconServer accompanied by the micro-CT system was used to reconstruct all resultant planograms under identical scanning parameters. The realistic three-dimensional (3D) visualization of knee joint specimens was performed by CTvox along with the micro-CT system.

**AFM Test of Surface Roughness of the Engineered or Regenerated Cartilage.** Inspired by the nanomechanical test reported previously,19 an atomic force microscope (AFM, Bruker Multimode8, USA) was employed to measure the surface modulus and the surface roughness of normal cartilage, neo-cartilage repaired under the 16-week implantation of cell-seeded, and virgin bilayered PLGA/PLGA-HAp scaffold. Prior to the AFM test, the cartilage slices with 2 mm in length and 2 mm in width were gently peeled in a thin layer from the experimental site, and the obtained cartilage slices were placed on the specimen stage. Thereafter, the PeakForce Quantitative NanoMechanics (PeakForce QNM) mode with the tip type of RTESP-300 (Bruker, USA) was carried out in the AFM test. Nanoscope Analysis from Bruker’s offline
data processing software was used to calculate the surface modulus and the surface roughness of the engineered cartilage specimens.

**Tissue Retrieval and Histological Analysis.** All experimental rabbits were sacrificed by injecting the excess pentobarbital sodium at 8 and 16 weeks postoperatively. After harvesting and photographing the knee joint specimens, some specimens were fixed in 4% formalin and prepared for the micro-CT observation. All remaining samples were fixed in 4% formalin, decalcified in 30% formic acid, embedded in paraffin, and sectioned at 4 μm by using a rotary microtome (Leica RM2235, Germany).

Some sections were stained with hematoxylin and eosin (H&E), toluidine blue, and safranin O. Moreover, the remaining sections were further collected for immunohistochemical assessment of collagen type II, which is the characteristic ECM component in hyaline cartilage.

**Western Blot To Detect the Relevant Protein Expression.**

Western Blot method was employed to determine the protein expression of p-smad 1, smad 2, collagen type I, and collagen type II. According to the manufacturer’s protocol, cells were rinsed by precooling PBS three times, and then cellular proteins were extracted with 400 μL mixture (1:100) of phenylmethanesulfonyl fluoride (PMSF) and RIPA lysis buffer (Medium, CoWin Biosciences Co., Ltd., China) under an ice bath. After 30 min, centrifugation was carried out at 12 000 rpm at 4 °C for 5 min, and subsequently, the supernatant was collected. Bicinchoninic acid protein assay kit (BCA, CoWin Biosciences Co., Ltd., China) was employed to detect the concentration of the target protein. The preprepared protein and SDS-PAGE loading buffer (reducing, 5X, CoWin Biosciences Co., Ltd., China) were mixed well in a proportion of 4:1 and then placed in the boiling water bath for 5 min. Thereafter, an expeditious ice bathing was carried out until it was cooled to room temperature. The sample was preserved at −20 °C after a transitory centrifugation.

According to the molecular weights of the target genes, a 10% gel was chosen to prepare the gelling system for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). PageRuler pre-stained protein ladder (#26616, 10 to 180 kDa, Thermo Scientific) and 40 μg of prepreparred protein sample conditioning fluid were added into the sample holders, and residual sample holders were added in the loading buffer (reducing, 1X). Electrophoresis voltage was kept at 90 V. When the blue protein bands appeared, electrophoresis voltage was changed to 120 V.

Thereafter, western transfer was carried out by a poly(vinylidene fluoride) (PVDF) membrane (Millipore, 0.45 μm) under the electric current of 200 mA for 70 min. 10% BSA was employed to seal the samples for 60 min. To detect the target proteins in the immunoprecipitate, SDS-PAGE and Western blot analyses were carried out with a 1:1000 dilution of primary antibody followed by a 1:5000 dilution of horseshardis peroixidase-conjugated antirabbit secondary antibody (Abcam).

The expression level of each gene of interest was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Bioworld) with a 1:1000 dilution overnight at 4 °C. Immunoprecipitated proteins were visualized by ECL reagent kit (Poinbio) with a 1:1 dilution in the chemiluminescence imager (3600, Clixn Science Instruments Co., Ltd. China). Western blot stripping buffer (CoWin Biosciences Co., Ltd, China) was used in a shaker for 30 min. The grayscale of Western blot bands were treated with a statistics software (Prism 5.0, GraphPad Software).

**Statistical Analysis.** All quantitative results are expressed as mean ± standard deviation. Data were analyzed with one-way analysis of variance (ANOVA) or Student’s t-tests to examine the difference between the two samples. Statistically significant differences are performed by using “*” for p < 0.05, “**” for p < 0.01, and “***” for p < 0.001.

## RESULTS

**Fabrication of Bilayered Composite Scaffolds.** We used room-temperature compression molding and porogen leaching to fabricate PLGA porous scaffolds. Thereafter, the interior surface of the bony-layer PLGA scaffold was modified by HAp deposition. Ultimately, the required bilayered PLGA/PLGA-HAp composite scaffolds were fabricated by gluing the cartilage and subchondral layer scaffolds by CH2Cl2, as schematically presented in Figure 1c.

Some as-fabricated cylindrical bilayered PLGA/PLGA-HAp scaffolds are shown in Figure 2a. The cylinder scaffolds were of diameter 4 mm and height of 5 mm, where the cartilage layer was of height of 1 mm, as demonstrated in Figure 2b. The cementing line of the bilayered PLGA/PLGA-HAp scaffolds is also indicated in Figure 2b. The upper and lower layers were connected very tightly in our bilayered porous scaffolds.

SEM was employed to observe the interior pore structure and morphology of the bilayered PLGA/PLGA-HAp scaffolds. The yellow dashed line in Figure 2c represents the cementing line or boundary between the upper cartilage layer and under the subchondral bone layer. Because a slight extrusion was performed when gluing the scaffolds, a few pores near the dashed line became squashed (Figure S1), or became compacted. The remaining pores had a rational pore sizes as we controlled with porogen. A magnified view (Figure S2) illustrates relatively smooth pore walls of the cartilage-layer scaffold and a lot of HAp deposition covering on the interior surface of the bony-layer scaffold.

The porosity and pore size were well controlled by the fraction and size of sodium chloride porogen. The resultant cartilage-layer scaffolds were of pore sizes 100–200 μm, while those of the subchondral bone layer were of pore sizes 300–450 μm.

We determined the porosities of cartilage-layer and subchondral-layer scaffolds by a modified liquid replacement method, as calculated by eq 1. The results are shown in Figure 3. The porosity of the cartilage layer scaffold was about 92%; the subchondral layer PLGA scaffold had a porosity of about 77%. The porosity of the PLGA scaffold with HAp modification was about 76%, which was calculated with eq 2.
The deposition on the interior surface of the subchondral bone layer scaffold was further characterized. FESEM-EDS were employed to qualitatively detect the calcium–phosphorus ratio of deposition. As demonstrated in Figure 4a, elements calcium and phosphorus were detected on the bony-layer PLGA/HAp scaffold. The peak area of each element was calculated, and the calcium–phosphorus ratio was obtained as 1.69, which was close to the theoretical calcium–phosphorus ratio of hydroxyapatite 1.67.69,70 Figure 4a shows also the images of the elements carbon, oxygen, calcium, and phosphorus, confirming the success of the deposition on the interior surfaces of scaffolds.

Natural HAp is an inorganic crystal.71 Thereby, XRD was employed to detect the diffraction peaks of the deposition on the interior surface of the subchondral-layer PLGA scaffold. We used a commercial HAp (Sinopharm Chemical Reagent Co., Ltd., China) as standard, and we proved that the deposition is a kind of analogous hydroxyapatite (Figure 4b). Thereinto, the characteristic diffraction peaks of HAp were observed at the 2θ angle of 27.4° and 31.8°. They were not as sharp as the characterized diffraction peaks of commercial hydroxyapatite, which illustrated that the deposited HAp was of just a moderate degree of crystallization.

Thereafter, TGA technology was employed, taking advantage of difference of heat stabilities of polymer and ceramics. The fraction of HAp deposition on the interior surfaces of the subchondral-layer PLGA scaffold was determined to be 13.9%, as shown in Figure 4c.

Mechanical Properties of Bilayered Scaffolds. The mechanical properties of the scaffolds at “dry” and “wet” state were also characterized. Figure 5a schematically shows the process of compression testing for the bilayered scaffold. Compressive modulus ($E$) is generally used to characterize the...
According to Figure 5c, the compression moduli of the indicated four groups of scaffolds at the "wet" state are shown in Figure S3. We also made normalization of the data at the "wet" state to those at the "dry" state with respect to each group, and then integrated the normalized values from all of the groups into Figure 5. Despite of hydrophobicity of PLGA, the "wet" moduli were significantly less than the "dry" data, consistent with our previous finding. The present study indicated that about 80% moduli remained after the wetting process. Since tissue engineering or tissue induction scaffolds are eventually used in a wet aqueous environment, such a "discount" should be taken into consideration in a biomaterial design based on merely "dry" mechanical data. Nevertheless, Figure 5 in the main manuscript and Figure S3 in Supporting Information exhibited similar trends of the modulus changes among different groups, and thus, "dry" or "wet" did not alter the basic conclusions of comparison between different scaffold groups with PLGA as the basic matrix.

**In Vitro Cell Adhesion on the Interior Surfaces of the Porous Scaffolds.** Besides physicochemical characterization of the scaffolds, we seeded the vigorous MSCs onto scaffolds for the cartilage layer (PLGA) and subchondral bone layer (PLGA or PLGA/HAp) to assess cell adhesion and proliferation *in vitro*. After a 7-day culture in the basal medium, MSCs were fixed on the interior surface of the scaffolds, and then F-actins and nuclei of MSCs were fluorescently stained. Thereafter, an inverted fluorescence microscope was employed to observe the immunofluorescence staining MSCs, with some images demonstrated in Figure 6.

Cells adhered on the interior surfaces of the porous scaffolds very well in all of groups. Besides these fluorescence micrographs in the main manuscript, a typical SEM image is shown in Figure S4, which demonstrated further satisfactory MSC growth on the interior surface of the scaffolds. Hence, we confirmed partially *in vitro* biocompatibility of both layers of our composite scaffolds.

**In Vivo Osteochondral Repair with a Rabbit Model.** In the *in vivo* experiments, a defect with 4 mm in diameter and 5 mm in depth was created in the media femoral condyle by applying a surgical drill bit, as demonstrated in Figure 7a. After one-week MSC culture on the cartilage layer of bilayered PLGA/PLGA-HAp scaffold, the cell-seeded and virgin bilayered PLGA/PLGA-HAp scaffolds were implanted into the rabbit knee joints to examine the efficacies of tissue engineering and tissue regeneration, respectively.

After 16 weeks, a macroscopic specimen examination of knee joints was carried out to make sure of no inflammation of the synovial membrane and other joint tissues. In the blank control group (Figure 7b), compared with the normal osteochondral site on the left, the defect on the right was covered with a few amorphous soft fibrous tissue around the interior walls, yet an obvious vacancy could still be seen in the lateral condyle at 16-week postoperation.

In the group of virgin bilayered PLGA/PLGA-HAp scaffolds (Figure 7c,e), the vast majority defects were filled with regenerated tissue after a 16-week implantation, and some bone trabeculae were found in the defect site via the micro-CT observation.
Figure 6. Fluorescence micrographs of MSCs after 7 days of culture in the basal medium on cartilage layer and subchondral layer scaffolds.

Figure 7. Observation of the osteochondral defects and repair effects in rabbit knee joints. (a) A defect was created in the media femoral condyle by applying a surgical drill bit; the defect was 4 mm in diameter and 5 mm in thickness in the medial condyles of the knee joint, and the medial condyle defect was implanted with scaffold; (b) the knee joint defects without implanting any scaffold after 16 weeks; (c) the knee joint implanted with PLGA/PLGA-HAp bilayered scaffolds after 16 weeks, and (e) its micro-CT observation; (d) the knee joint implanted with PLGA/PLGA-HAp bilayered scaffolds with MSCs after 16 weeks, and (f) corresponding micro-CT images.
In the cell-seeded bilayered PLGA/PLGA-HAp scaffold group (Figure 7d,f), all defects were repaired completely with a cartilage-like tissue, which was smooth as the normal cartilage, and the demarcation integrated with the surrounding tissue was not visible. The bone trabeculae were uniformly distributed in the defect via the micro-CT observation, indicating repair of cartilage and subchondral bone simultaneously.

Biomechanical Tests as well as Surface Roughness Observations of Regenerated Cartilage with AFM. The biomechanical property of the 16-week regenerated cartilage is a significant proof for assessing the repair ability. Considering that it is not easy to dig out the regenerated tissue and make its shape regular from the defect site, we employed AFM to measure the surface modulus and the surface roughness of the regenerated cartilage on the appropriate length scales.

Figure 8a,b show the distribution of surface modulus and roughness of the normal and regenerated cartilages. The Young’s moduli of 16-week regenerated cartilage of the cell-seeded bilayered PLGA/PLGA-HAp scaffold, the virgin bilayered PLGA/PLGA-HAp scaffold, and normal cartilage were, respectively, in a range of 6–10, 3–9, and 7–11 GPa, which is consistent with the data for joint cartilage.19

In Figure 8b, nanoscope analysis resulted in the surface roughness of 16-week regenerated cartilage of the cell-seeded bilayered PLGA/PLGA-HAp scaffold, virgin bilayered PLGA/PLGA-HAp scaffold, and normal cartilage. They read 10.4 ± 2.6 nm, 19.6 ± 5.3 nm, and 8.4 ± 0.4 nm, respectively. Taking all the above data into consideration, it is conclusive that the virgin bilayered PLGA/PLGA-HAp scaffold repaired most of the cartilage defect, and the seeding of external cells made some additional improvement.

Histological Examinations of the Regenerated Tissues To Show Characteristics of Cartilage and Subchondral Bone. To assess the tissue response to the implanted bilayered scaffolds and the defect-repairing progress, histological examinations were carried out. No significant inflammatory response was observed in the histological examination among all of groups.

In the blank control group, the staining results showed just a small amount of irregular fibrous tissues in the subchondral region, and the obvious vacancy could still be seen at 16-week postoperation, as demonstrated in Figure 9a.
PLGA-HAp group, most of the defects were padded with regenerated tissue mixed with immature cartilage in the chondral layer, and new bone was formed in the subchondral region. Hence, our bilayered PLGA/PLGA-HAp scaffold triggered a significant healing progress although a handful of residual scaffold was still perceived in the histological examination after a 16-week implantation. In the group of PLGA/PLGA-HAp with MSCs, the defects were filled almost completely after 16 weeks. A higher percentage of hyaline cartilage in the chondral layer and the bone regeneration in the subchondral region, which were well integrated with native tissue in the site.

The high-magnification observations after H&E, safranin O, and toluidine blue staining demonstrated that ECM was actively secreted in the virgin and cell-seeded groups. Oval-shaped cartilage lacuna was clearly formed, which is a very important characteristic of hyaline cartilage (Figure 9b).

Another critical characteristic of cartilage is the expression of type II collagen. Immunohistochemical tests were thus employed to detect the expression of such a type of collagen in the specimen sections of the virgin and cell-seeded groups, as shown in Figure S5. While a slightly lower expression level of type II collagen was found for the virgin group, both groups exhibited similar expression levels of type II collagen to the normal group.

**Western Blot Analysis of Expression of Characteristic Proteins in the Regenerated Tissues Extracted from the Implanting Sites of the Bilayered Composite Scaffolds.** Western blot was used to semiquantify the target protein expression in neo-tissues repaired by the virgin and cell-seeded bilayered PLGA/PLGA-HAp scaffolds after a 16-week implantation. Thereinto, protein expressions of p-smad 1, smad 2, collagens (type I and type II) were determined (Figure 10). In both the virgin group or cell-seeded group, the relevant
proteins remained on an upward expression from 8 weeks to 16 weeks. As for the relative level of proteins p-smad 1, smad 2, type I collagen, and type II collagen, we observed a higher expression in the cell-seeded PLGA/PLGA-HAp group than in the virgin group. There is an inversion of the expression levels of type II collagen between the virgin and cell-seeded groups at 16 weeks.

**DISCUSSION**

**Tissue Regeneration or Tissue Induction: Like Tissue Engineering, an Important Strategy To Regenerate Cartilage and Subchondral Bone Based on Appropriate Scaffolds.** While the concept of tissue engineering using porous scaffolds and external seed cells to engineer a tissue is a milestone in medicine, tissue regeneration or tissue induction is another strategy using only implanted biodegradable and bioactive materials and recruiting internal cells. Both strategies are very helpful for regenerative medicine. While much progress has been made in the tissue regenerative biomaterials, the present study develops a facile biomaterial technique to prepare a bilayered composite scaffolds composed of two FDA-approved biomaterials, a biodegradable polymer PLGA and a bioactive ceramic HAp.

The repair of articular cartilage defects remains a rock-ribbed challenge considering that natural articular cartilage lacks self-healing capacity and ideal clinical treatments. Tissue engineering and regenerative medicine emerged as an alternative strategy with great promise for articular cartilage regeneration. Driven by increasingly clinical needs, there have been many studies on articular cartilage repair. In recent years, bilayered porous scaffolds have been raised to simultaneously mimic interior structures and physiological properties of cartilage and subchondral bone. Cell-seeded bilayered scaffolds probably have a better repairing capacity than the cell-free ones. However, the introduction of extra cells led to much restriction such as long-time preparation, complicated examination, and expensive regulatory approval of applications because of the cost and the immunogenic problems. The regulatory disadvantages thereby enhance interest in virgin bilayered scaffolds for articular cartilage repair. The mechanical properties and interior structural optimization are still a big challenge for those virgin bilayered scaffolds. So we are highly interested in appropriate bilayered scaffolds for osteochondral tissue engineering and tissue regeneration.

**Bilayered PLGA/PLGA-HAp Composite Scaffold: Affording One of the Appropriate Biomaterial Systems.** In this study, a bilayered PLGA/PLGA-HAp composite scaffold was fabricated by combining room-temperature compression molding and particle leaching, plasma-treated surface deposition, and solvent sticking methods to simultaneously regenerate cartilage and subchondral bone. PLGA and HAp are both approved by the FDA for clinical applications.

In addition to the bilayered design and elaborate material selection, porosity and pore size of a bilayered scaffold were well controlled by the fraction and size of porogen. As reported previously for tissue engineering, the cartilage layer scaffold with a porosity of 92% and a pore size of 100–200 μm are thought to promote chondrogenesis, and the subchondral bone layer scaffold with a porosity of 77% and a pore size of 300–450 μm are recommended for promoting vascularization and bone formation. These pore sizes and porosity were taken in the present study.

We succeeded in fabrication of the expected bilayered PLGA/PLGA-HAp composite scaffolds, as demonstrated in Figures 2 and 3. The mold was designed at a diameter of 4 mm, and there was an un conspicuous scaffold contraction because of solvent evaporation. If we have a close-up view of the cementing line between the cartilage layer and the subchondral layer, it is not difficult to observe that a few pores became squashed and even had compaction at a thickness of about 85 μm (Figure S1), due to compression when upper and down layer scaffolds were glued. This slight squashing and compaction made the two layers connected tightly and became a positive influence factor of mechanical properties for the bilayered scaffolds. As shown in Figure 5, the combined efficiency of the mechanical properties of bilayered scaffolds.

![Figure 10. Western blot analysis. (a) Protein bands presentation of p-smad 1, smad 2, collagen type I, and collagen type II; (b) analysis of gray scale values of those protein expression in Western blot. The asterisk indicates significant difference between the examined two groups. n = 4 for each group.](image-url)
scaffolds was greater than the sum of each single-layer scaffold used in isolation.

MSCs have recently aroused the concern of fundamental research.97,98 For the in vitro study of biocompatibility, F-actins and nuclei of 7-day cultural MSCs in the bilayered PLGA/PLGA-HAp scaffolds were fluorescently stained, and we did observe a significant cell growth in the porous scaffolds. The New Zealand white rabbit model was chosen for our in vivo experiments. MSCs were employed to inject onto the cartilage layer. As for the cell-seeded bilayered PLGA/PLGA-HAp scaffold group, the 16-week follow-up results indicated a good spontaneous healing of the osteochondral defect on the biomechanics and morphological character, as shown in Figures 7, 8, and 9.

After a 16-week implantation of the virgin bilayered PLGA/PLGA-HAp scaffold into the osteochondral defect, most of the repair was achieved. Additionally, the scaffold seeded with MSCs led to an additional repair. Both the groups with porous scaffolds behaved much better than the untreated group. With respect to the proportion and biomechanics of regenerated tissue and the morphology of neo-chondrocytes (Figures 7, 8, and 9), osteochondral defects treated with a virgin bilayered PLGA/PLGA-HAp scaffold alone or a bilayered PLGA/PLGA-HAp scaffold associated with MSCs showed a similar cartilaginous and subchondral formation. Therefore, it is the bilayered PLGA/PLGA-HAp scaffold that mainly contributed to the progress of cartilage and subchondral bone regeneration. This finding is consistent with some previous studies.99,100

In addition, Western blot was employed to semiquantify the target protein expression of p-smad 1, smad 2, collagen type I, and collagen type II in neo-tissue repaired by the cell-seeded and virgin bilayered PLGA/PLGA-HAp scaffolds after a 16-week implantation (Figure 10a). It has been known that the levels of gene expressions of p-smad 1 and type I collagen were positively related to osteogenesis;101−103 it has also been reported that the levels of gene expressions of smad 2 and type II collagen were positively related to chondrogenesis.104,105 As shown in Figure 10b, the levels of the relevant protein expression in the virgin group and cell-seeded group remained on a consistent and upward trend. Although most protein expression had a higher level for the cell-seeded group, there was a slight inversion on the level of protein expression of type II collagen in 16-week implantation of virgin bilayered PLGA/PLGA-HAp scaffolds, indicating the potential of the composite bilayered scaffold in osteochondral regeneration.

**Composite Scaffold: The Biomechanical and Chemical Microenvironment Mimics Cartilage and Subchondral Bone.** There are different moduli, physical structures, and physiological functions between articular cartilage and subchondral bone; therefore, we mimicked their biomechanical requirements to fabricate a bilayered composite scaffold with different porosities and pore sizes. Moreover, the mechanical properties under the “wet” state as simulated physiological environment should be taken into consideration, especially for the biomaterial design of in situ tissue induction. In this study, the mechanical properties of cartilage-layer PLGA scaffold and subchondral-layer PLGA/HAp scaffold of our bilayered scaffold were tested at their “dry” and “wet” states, and the results showed a befitting biomechanical matching for the as-fabricated bilayered composite scaffold.

According to the studies reported previously, the instantaneous compressive Young’s modulus of articular cartilage is 1.36−39.2 MPa, and the compressive modulus of subchondral bone is 1.4−9800 MPa.27,106,107 The cartilage layer made of PLGA and the subchondral bone layer made of PLGA/HAp in the bilayered scaffold at the “wet-state” had moduli of 2.8 ± 1.7 MPa and 20.3 ± 0.7 MPa, respectively. Our bilayered PLGA/PLGA-HAp composite scaffolds exhibited biomechanic matching.

Moreover, we also tested the biomechanical properties of 16-week regenerative articular cartilage, showing an applicable result with a comparison of normal articular cartilage. Obviously, a favorable biomechanical environment afforded not only a supportive interconnective structure but also a biomimetic mechanical stimulation, which is greatly beneficial to the growth of cells and tissues.

It has been reported that surface chemistry and physics of scaffolds can prominently regulate cell behaviors.108−111 One of our previous works68 illustrated that chemical functional groups on the surface of biomaterials can regulate stem cell differentiation via tuning protein adsorption and then non-specific cell adhesion and thus cell spreading. We think that the HAp deposition on the interior surface of scaffold for the subchondral bone might be helpful for cell adhesion, differentiation, and bone matrix formation.48,49,77,79,112 MSCs could be triggered to facilitate the formation of tissue cells for matching the microenvironments.74,22 MSCs seeded into the cartilage layer and subchondral layer can facilitate the formation of cells toward cartilage and subchondral bone, respectively. Our HAp coating can afford a better surficial chemistry microcircumstance to drive the seed cells into the subchondral bone differentiation. It is thus reasonable that the cartilage and subchondral bone were restored well under the implantation of the cell-seeded bilayered PLGA/PLGA-HAp scaffold.

A converse speculation is not hard to be made. If the tissue induction method is employed for osteochondral repair, even though exogenous seed cells are not used, endogenous chondrocyte, osteocyte and MSCs are presumably guided to migrate into the defect sites, and MSCs might be triggered to facilitate the formation of chondrocytes and subchondral bone cells. New tissues could be formed along with the secretion of the corresponding ECM and the appearance of the corresponding histological morphology. Taking all the results using the virgin scaffolds into consideration, it is likely that this tissue regeneration process was mediated by MSCs recruited from the subchondral bone penetrating into the scaffolds toward autologous matrix-induced chondrogenesis, similar to a previously proposed “cell homing” concept.113−117

**Limitations of the Present Studies.** Several limitations in this study have to be mentioned. Only a 16-week follow-up period was examined after the implantation in vivo, so that the results obtained by using merely the porous scaffold cannot have an identical repair effect to the cell-seeded group. Another critical argument comes from the probable resorption of neo-cartilage or neo-bone formed via tissue engineering or tissue regeneration after 3−5 years.118−120 Thus, a long-term follow-up study of outcomes is needed for the newly regenerated tissue, in particular, to examine the phenotype of chondrocytes and the biomechanical properties of the tissue. The larger animal model, which closely mimics the loading conditions of human knee joints, are also necessary to be considered.121,122

Anyway it is attractive if the bilayered scaffold has the ability to induce osteochondral tissue reestablishment without necessarily seeding cells. First, the biological and ethical problems related to the cells culture would be no longer in...
existence. Meanwhile, free of cells helps a porous scaffold tremendously cut its costs. More importantly, it could be fabricated as an off-the-shelf and easy-to-use product for clinical applications.

**CONCLUSIONS**

Bilayered PLGA/PLGA-HAp composite scaffolds were fabricated after combination of room-temperature compression-molding/porogen-leaching to prepare PLGA scaffolds for each layer of the cartilage and the subchondral bone, the bioceramic deposition of the interior pore layer to obtain the PLGA-HAp scaffold for the subchondral bone layer, and finally to use an organic solvent to stick the two layers. The bilayered PLGA/PLGA-HAp composite scaffold was found to repair an osteochondral defect with the simultaneous formation of cartilage and subchondral bone both with and without MSCs as seed cells. While the introduction of the extra cells led to better results, the bilayered composite scaffold exhibited satisfactory in vivo efficacy after being implanted into the rabbit knee joints for 16 weeks. According to the biomechanical analysis, micro-CT observations, histological observations, and relative expression levels of characteristic proteins via Western blot, the bilayered PLGA/PLGA-HAp composite scaffold exhibited a favorable regeneration of osteochondral defect. The biomechanics of neo-tissues characterized by AFM basically matched with that of native tissue. This study illustrates that the bilayered PLGA/PLGA-HAp scaffold is appropriate for osteochondral tissue engineering and tissue regeneration, and even a tissue induction after implanting the composite scaffolds without seeding cells is available for in vivo osteochondral regeneration.

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**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.8b00552.

SEM images to present the high-magnification border of the cartilage and subchondral layers; SEM observations of the subchondral bone layer before and after HAp coating on the interior surfaces of PLGA scaffolds; SEM images of MSCs on the scaffolds after 7-day cell culture; mechanical properties of the as-fabricated PLGA porous scaffolds at “dry” state and the normalized moduli of the as-fabricated four groups of scaffolds at both “dry” and “wet” states; histological images of reparative tissues after 16-week implantation of bilayered PLGA/PLGA-HAp scaffolds alone and seeded with MSCs (staining of type II collagen) (PDF)

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