Bony defect repair in rabbit using hybrid rapid prototyping polylactic-co-glycolic acid/β-tricalciumphosphate collagen I/apatite scaffold and bone marrow mesenchymal stem cells

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Abstract

Background: In bone tissue engineering, extracellular matrix exerts critical influence on cellular interaction with porous biomaterial and the apatite playing an important role in the bonding process of biomaterial to bone tissue. The aim of this study was to observe the therapeutic effects of hybrid rapid prototyping (RP) scaffolds comprising polylactic-co-glycolic acid (PLGA), β-tricalciumphosphate (β-TCP), collagen I and apatite (PLGA/β-TCP-collagen I/apatite) on segmental bone defects in conjunction with combination with bone marrow mesenchymal stem cells (BMSCs).

Materials and Methods: BMSCs were seeded into the hybrid RP scaffolds to repair 15 mm defect in the radius of rabbits. Radiograph, microcomputed tomography and histology were used to evaluate new bone formation.

Results: Radiographic analysis done from 12 to 36 weeks postoperative period demonstrated that new bone formed at the radial defect site and continues to increase until the medullary cavity is recanalized and remodelling is complete. The bone defect remained unconnected in the original RP scaffolds (PLGA/β-TCP) during the whole study. Histological observations conformed to the radiographic images. In hybrid RP scaffold group, woven bone united the radial defect at 12 weeks and consecutively remodeled into lamellar bone 24 weeks postoperation and finally matured into cortical bone with normal marrow cavity after another 12 weeks. No bone formation but connective tissue has been detected in RP scaffold at the same time.

Conclusion: Collagen I/apatite sponge composite coating could improve new bone formation *in vivo*. The hybrid RP scaffold of PLGA/β-TCP skeleton with collagen I/apatite sponge composite coating is a promising candidate for bone tissue engineering.

Key words: Apatite, bone tissue engineering, collagen I, hybrid scaffolds, segmental bone defect

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Introduction

Theoretically, bone tissue can regenerate and bone defects can completely fill. Nevertheless, when a bone defect cannot heal through its normal wound healing response, surgical intervention is required to reconstruct the defect site with various forms of grafts and implants. Currently, autografts, allografts and artificial biomaterials are commonly used to replace the missing tissue. Autologous cancellous bone grafting is still the gold standard.[1] However, donor site morbidity and limited graft availability push clinicians and researchers to use allograft bones and artificial biomaterials as alternatives.[2,3] For allografts, possible pathogen transfer and degradation of the tissue's material properties, bone mineral density and prevalence of microcracks is a major concern.[4,5] Biomaterials do not face similar problems and therefore, hold considerable promise. There are several kinds of materials, which have

been designed into experimental and clinical uses including natural ingredients (collagen, coral), synthetic polymers (polylactic acid, polyglycolic acid and their copolymers), bioceramics (hydroxyapatite, tricalciumphosphate). However, a single material may hardly cater to the increasing demands of biocompatibility, osteoconductivity, biodegradation and mechanical property that the implanted scaffold should have in practical cases. Now-a-days, integrated hybrid biomaterials hold more promise than a single material.

As reported in an earlier study[6] we developed a three dimensional porous hybrid biomaterial consisting of PLGA, β-tricalciumphosphate (β-TCP), collagen I and apatite, namely the PLGA/β-TCP-collagen I/apatite scaffold. As the skeleton of this scaffold has been designed and produced by rapid prototyping (RP) technology, with low temperature deposition manufacturing (LDM) method, the scaffold was biodegradable, highly porous (>90%) with interconnected macro pores of about 400 μm and micropores less than 10 μm, was mechanically strong and could be easily forged into desired shapes.[7] However, there still remain problems to be solved not only relating to its hydrophobic surface, which is unfavorable for cell attachment and osteogenic development, but also relating to its decreased bone bonding property caused by the PLGA ingredient. To address those problems, the PLGA/β-TCP skeleton has been hybridized with collagen I and apatite. Earlier data demonstrated that the novel scaffold could improve cell proliferation and promote osteogenic differentiation with hydrophilic surfaces *in vitro*. However, the reconstructive ability of the hybrid scaffolds remains unknown *in vivo*. The purpose of the current study was to evaluate whether the hybrid scaffolds combined with bone marrow mesenchymal stem cells (BMSCs) can enhance bone defect repair in a rabbit model compared with the PLGA/β-TCP skeleton.

MATERIALS AND METHODS

Preparation of PLGA/β-TCP skeleton and the PLGA/β-TCP-collagen I/apatite hybrid scaffold

PLGA/β-TCP (7:3 w/w) skeleton was fabricated by a LDM system (Tissform 3, the Department of Mechanical Engineering of Tsinghua University) as we reported before.[7] Briefly, the hybridization procedure has been accomplished as follows: firstly, PLGA/β-TCP was immersed in collagen I (derived from fetal bovine corium) acidic solutions (pH 3.2, 0.5 wt.%) under vacuum for 4 h, then freeze-dried at −80°C under a vacuum of 0.2 Torr for an additional 72 h to form collagen microsponges in the skeleton pores. The new formed collagen microsponges were further crosslinked by treatment with glutaraldehyde vapor saturated with 25% glutaraldehyde aqueous solution at 37°C for 4 h. After that,

the scaffolds were incubated in a simulated body fluid (SBF) (50 ml in volume with pH 7.4 including 141 mM NaCl, 4.0 mM KCl, 0.5 mM $MgSO_4$, 1.0 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 2.5 mM $CaCl_2$ and 1.0 mM KH_2PO_4, deionized water) for up to 16 days at 37°C to form mineral film. The SBF solution was changed every 24 h to ensure sufficient ion concentrations for mineral growth.[6]

Marrow Mesenchymal Stem Cells culture and combination with the hybrid scaffolds

The animal study was approved by the Institutional Animal Review Committee of the Fourth Military Medical University. BMSCs were obtained from the os longum of 1-day-old New Zealand white rabbit and cultured under Dulbecco's modified Eagle's medium (DMEM; Gibco) plus 10% fetal bovine serum (FBS) at 37°C, 5% CO_2. The medium was changed every 3 days. Cell subcultures of third passages were osteogenically induced under DMEM supplemented with 10% FBS, 10^{-8} mol/L dexamethasone, 10 mM β-glycerolphosphate and 50 mg/ml L-ascorbic acid for 3 weeks.[6] Then the BMSCs were trypsinized and suspended into each $15 \times 3 \times 3$mm sized hybrid scaffolds at a density of 7×10^7/ml to fabricate BMSCs-PLGA/β-TCP-collagen I/apatite composite and incubated for another 24 h waiting for implantation. In addition, the BMSCs/PLGA/β-TCP composite was prepared in the same way as control.

Operative procedure

48 healthy New Zealand White rabbits weighing between 2.5 and 3.5 kg were used in this study. Rabbits were anesthetized with a cocktail of 50 mg/kg ketamine and 8 mg/kg xylazine administered intramuscularly. The operation sites over radii were exposed after skin incisions. An approximately 2.5cm long incision was made and the tissues overlying the distal diaphyseal radius were dissected. A 15 mm osteoperiosteal defect was created in the radius with a mini-oscillating saw bilaterally. The PLGA/BTCP hybrid samples were inserted in the defect without external fixation. For experimental groups, the radial defects were implanted with BMSCs-PLGA/β-TCP-collagen I/apatite composites (group A, $n = 30$), BMSCs/PLGA/β-TCP composites (group B, $n = 30$), PLGA/β-TCP-collagen I/apatite hybrid scaffolds (group C, $n = 15$) and PLGA/β-TCP scaffolds (group D, $n = 15$). Six additional defects remained untreated as blank control (group E, $n = 6$). A balanced split-plot randomization scheme was used to locate the scaffolds so that the limb choice or formulation pairing would not influence the outcome. All surgeries were conducted by the same investigator. After surgery, animals were radiographed immediately. Buprenorphine (0.05 mg/kg) and enrofloxacin (50 mg/kg) was given for pain relief and infection prophylaxis. Water and food were supplied *ad libitum*.

Evaluation of results

At 12, 24 and 36 weeks postsurgery, animals were euthanized with an over-dose of pentobarbital. All bone defects and surrounding tissue were retrieved en bloc. Radiographs image were undertaken immediately. Furthermore, each radiograph was examined by two independent observers and given a score depending on a standardized scoring system including an evaluation of bone formation (5 levels), persistence of the fracture line (3 levels) and bone remodeling (3 levels).[8] Meanwhile, microcomputed tomography (microCT) (eXplore Locus SP MicroCT, GE company American) was also done for three-dimensional (3D) reconstruction of the original orthotopic defect sites in different groups postoperation.

The specimens so obtained were preserved in 4% buffered formaldehyde, dehydrated and embedded in paraffin. Serial sections were stained with hematine/eosine (HE) and observed by light microscopy (Leica, Rijswijk, The Netherlands). As for morphometric analysis, five sequential sections per composite were selected for evaluation under low magnification, allowing coverage of the entire composite. Using a Leica-Qwin 3.2 image analysis system (Leitz DMRD, Leica Microsystems, Inc., Bannockburn, IL, USA), all slides were seen by two independent observers to identify the type of bone tissue. The extent of bone formation was indicated by the percentage of the bone tissue area within the defect site and an average value was calculated for each composite. Data were then averaged across all implants within each group.

Statistical evaluation

Quantitative data were expressed as mean value ± standard deviation and Student *t*-test was used to analyze the percentage of bone formation. Data of radiographic scoring were processed as ordinal ranking and analyzed with Mann-Whitney *U* test. Differences at $P < 0.05$ were regarded to be statistically significant.
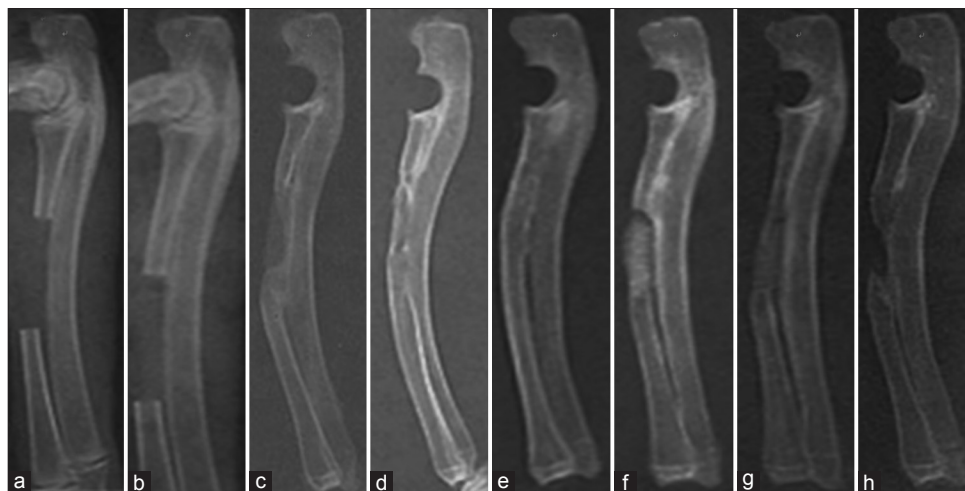
RESULTS

Macroscopic observations

Forty six rabbits were evaluated in the specified time point in this experiment. One in group A and the other in group C died during surgery. No sign of infection was detected in any rabbit until the harvest time.

X-ray analyses

X-rays of bone defects at operation day, 12, 24 and 36 weeks after implantation were analysed [Figure 1a]. Immediately after the operation, the composites in different groups were nearly invisible because of the low X-ray attenuation coefficient [Figure 1b]. When bone defect was treated with hybrid scaffolds (group A), new bone tissue appeared at the proximal and distal ends of the radial defect sites 12 weeks postoperation [Figure 1c]. In addition, the distance between both broken sides shortened and seemed almost united in the middle site [Figure 1c]. Another 12 weeks later, bone union was completely accomplished and bone medullary cavity was nearly recanalized. The radii began to rebuild and mold [Figure 1d]. 36 weeks after the operation, the remodeling of bone contour ended [Figure 1e]. The 15 mm segmental osteoperiosteal defect was repaired successfully. No radiographic sign of bone formation was presented at the defect sites in group B during the whole experimental period and the bone remained un-united 36 weeks postoperation [Figure 1f-h]. In groups C and D, which have not been combined with seed cells, the bone defect sites failed to repair 36 weeks after operation (data not shown). The radial bone defect in blank control group E was observed throughout

Figure 1: Radiographic results: (a) Bone defect caused by osteotomy; (b) radiograph immediately after scaffolds implantation; (c-e) 12, 24 and 36 weeks postsurgery of the bone marrow mesenchymal stem cells (BMSCs)-polylactic-co-glycolic acid (PLGA)/β-tricalciumphosphate (β-TCP)-collagen I/apatite composites; (f-h) 12, 24 and 36 weeks postsurgery of the BMSCs-PLGA/β-TCP composites

the study. The X-ray image scores of group A were 5.12 at 12 weeks, 9.68 at 24 weeks and 11.86 at 36 weeks with full marks of 12 levels while groups B-D have no scores from the beginning to the end ($P < 0.05$).
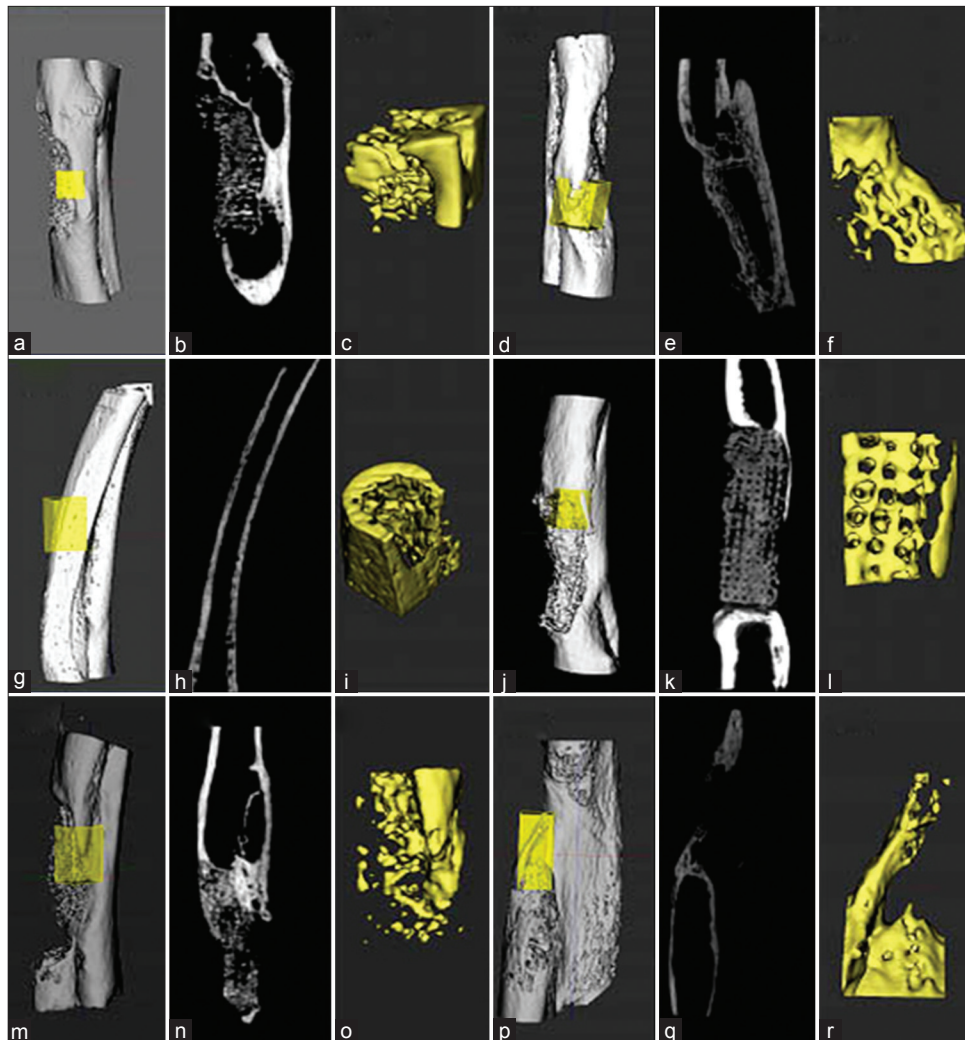
Micro CT results

Micro CT was used to demonstrate 3D images of the bone defect area as well as to determine the quantity of newly formed bone. In group A, new bone along the lateral edge of the residual scaffolds was seen after 12 weeks [Figure 2a-c]. Bilateral bone cortices formed with nearly recanalized marrow cavity [Figure 2d-f] after 24 weeks. After 36 weeks, the newly formed bone had a macroscopic structure and a CT value similar to that of the original radius with well remodeled bony outline [Figure 2g-i]. Meanwhile, no bone formation was detected after 12, 24 and 36 weeks

postoperation in group B except for the gradual degradation of implanted scaffolds until complete absorption [Figure 2j-r].

Histology

In the hybrid scaffolds group, the growing woven bone fully bridged the defect at 12 weeks after surgery. After 24 weeks, the woven bone remodeled into lamellar bone and bone marrow cavity began to form. Solid union was documented by the transition from dense lamellar bone to dense cortical bone with normal marrow cavity in the defect site 36 weeks postsurgery [Figure 3a-c]. In the original scaffolds group, no apparent bone tissue but remaining scaffolds after continual degradation were easily detected by light microscopy. Finally, the scaffolds degraded completely and were replaced by connective tissues [Figure 3d-f]. Percent of bone forming area in group A was $48.56 \pm 3.12\%$ at

Figure 2: Microcomputed tomography results: 1. Bone marrow mesenchymal stem cells (BMSCs)-polylactic-co-glycolic acid (PLGA)/β-tricalciumphosphate (β-TCP)-collagen I/apatite composites (a-i): 12 weeks postoperation showing new bone along lateral edge of residual scaffolds (a-c); 24 weeks postoperation showing nearly recanalized marrow cavity (d-f); 36 weeks postoperation showing remodelling (g-i); 2. BMSCs-PLGA/β-TCP composites (j-r): 12 weeks postoperation (j-l); 24 weeks postoperation (m-o); 36 weeks postoperation (p-r). 3D reconstruction image (a, d, g, j, m, p); reconstruction image in the coronal plane (b, e, h, k, n, q); magnified view (c, f, i, l, o, r) of yellow cubic area (a, d, g, j, m, p) showing no new bone formation
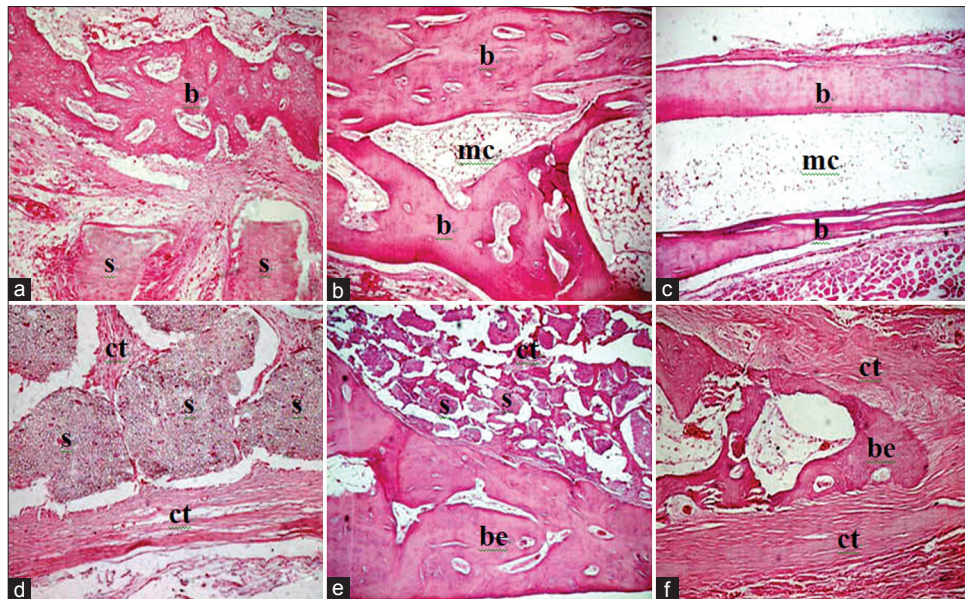
Figure 3: Histological results: 1. Bone marrow mesenchymal stem cells (BMSCs)- polylactic-co-glycolic acid (PLGA)/β-tricalciumphosphate (β-TCP)-collagen I/apatite composites (a-c): 12 weeks postoperation (a); 24 weeks postoperation (b); 36 weeks postoperation (c); 2. BMSCs-PLGA/β-TCP composites (d-f): 12 weeks postoperation (d); 24 weeks postoperation (e); 36 weeks postoperation (f). b = Bone tissue, s = Scaffold, mc = Medullary cavity, ct = Connective tissue, be = Broken end of radius

12 weeks, $78.34 \pm 2.85\%$ at 24 weeks and $97.27 \pm 2.65\%$ at 36 weeks, which was significantly superior to no bone occupation of groups B-D ($P < 0.05$).

DISCUSSION

In this study, we used a novel bioengineering composite by combining PLGA/β-TCP-collagen I/apatite hybrid scaffold with BMSCs and implanted these into critical-sized (15 mm) segmental bone defects in a rabbit model. The bone defect was successfully repaired by 36 weeks.

Bone tissue engineering, temporary three-dimensional scaffolds play an important role in the manipulation of functions of bone forming cells and guidance of the formation of the new bones into desired shapes. Generally, suitable scaffold should possess high porosity with a large surface-to-volume ratio and required hydrophilic surface properties that permit cell adhesion, differentiation and proliferation on one hand and adequate mechanical integrity to maintain the predesigned tissue structures on the other.[9,10]

Nevertheless, very low number of seeded cells could remain on the surface of various kinds of biomaterials (including autograft and allografts) under standard cell culture conditions. Most cells are lost due to the characteristics of surface free energy, namely the hydrophobicity of biomaterials. In order to enhance cell seeding efficiency and modulate cell-scaffold interactions, Extracellular matrix (ECM) which critically affects cellular adhesion, proliferation and differentiation has been researched. Previous studies have explored long chains of ECM proteins such as fibronectin (FN), vitronectin (VN) for scaffold surface modification. Biomaterials, which have been coated with these proteins have been proved to promote BMSCs adhesion, proliferation and osteogenic potentiality to unite segmental bone defect.[11-13] Mediated by cells' integrins and ECM adhesion proteins, cells are continuously subjected to nanotopographical cues.[14] As for bone tissue, the organic component type I collagen and inorganic component nanostructure hydroxyapatite constitute the main microenvironment. As far as biomimics theory is concerned, bone bioengineering scaffold should possess the similar property as natural bone presents. Therefore, the seeded cells may recognize the artificial nanotopography as natural milieu to a greater extent, leading to well integration with biomaterial and expectable bone formation *in vitro* and *in vivo*.

Type I collagen is essential for bone formation and matrix production in bone. It is expressed from the early stages of osteogenic differentiation and forms fibrils with a typical banding pattern of 68 nm width, a 3-5 nm banding depth and a 35 nm inter fibrillar spacing depth.[14] By interacting with its natural nanoenvironment, type I collagen acts as a major regulator of cell osteogenic differentiation and can also facilitate bone deposition of *in vitro* cultured stromal cells.[15] Similar to FN and VN, collagen I also contains cell binding peptides and enhances cell attachment and osteogenesis in biomaterials by surface coating process.[16] Considering the superiority of type I collagen over FN and VN, we

hybridized PLGA/β-TCP with collagen I fibers to enhance the hydrophilic property of scaffold surface.

Compared to pure β-TCP scaffolds, addition of PLGA achieved improved control of design parameters such as porosity, degradability, mechanical properties and varying degrees of pore size and shape for the composite scaffold. At the same time, the bioactive β-TCP content is largely contained within the bulk of the PLGA/β-TCP scaffold, rather than at the surface. Since the interactions between biocomposite and bone tissue occur first at the pore surface, the non-exposed β-TCP is in effect wasted. In order to enhance osteoconductivity of the scaffold, methods of surface modifications have been suggested in this study. Bioactive materials have been reported to bond to bone *in vivo* via formation of an apatite layer in the interface.[17] Furthermore, the calcium ions (Ca^{2+}) in the apatite layer are thought to play a key role. It is reported that the osteogenic proteins such as osteocalcin and osteopontin which are involved in the bone tissue bonding to materials surfaces are also Ca^{2+} binding proteins.[18] Similarly, to strengthen the bone-bonding ability of non-bioactive materials, biomimetic apatite coating methods involving the immersion of material substrates in SBF containing Ca^{2+} at physiological temperatures have been developed.[19] The apatite coating can promote bone ingrowths, enhance direct bone contact,[20,21] and facilitate differentiation of BMSCs along osteogenic lineage.[22] In natural bone, hydroxyl apatite is deposited in a regular manner on the collagen matrix. In order to mimic this unique structure, we developed a hybrid scaffold of PLGA/β-TCP skeleton, collagen I and apatite by depositing apatite particulates on the collagen I microsponge surfaces. The use of PLGA/β-TCP skeleton as a mechanical skeleton advanced the formation of the hybrid scaffold with desired shapes and gave the hybrid scaffold good mechanical properties. As the primary components of bone ECM, collagen I and apatite should provide the novel hybrid scaffold with satisfactory cell interaction and osteoconductivity *in vivo*.

Histological and radiological results in our study clearly illustrated the statistical difference of osteogenic potency between BMSCs-PLGA/β-TCP-collagen I/apatite composites and BMSCs-PLGA/β-TCP composites. Continuously active bone regeneration in the radial defects was found in the hybrid scaffolds group with the implantation time prolonged. The contact of bone to the material was intimate and direct without intervention of soft tissue. Within 36 weeks postimplantation, new bone density was almost equal to that of the host bone, which indicated complete osteointegration of the defect area. While in BMSCs-PLGA/β-TCP composites group, connective tissues appeared between the native bone and implanted composites and

no new bone tissue formed during the study. Inconsistent development status concerning bone and connective tissues as demonstrated in two cellular composites groups should be attributed to the different scaffold handling process.

To conclude, the results of reconstruction of rabbit's radial defect has been very encouraging with composite constituted by PLGA/β-TCP-collagen I/apatite scaffold and BMSCs. We therefore, propose the novel hybrid scaffolds be useful in bone tissue engineering.

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