The bone formation *in vitro* and mandibular defect repair using PLGA porous scaffolds

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Abstract: Highly porous scaffolds of poly(lactide-*co*-glycolide) (PLGA) were prepared by solution-casting/salt-leaching method. The *in vitro* degradation behavior of PLGA scaffold was investigated by measuring the change of normalized weight, water absorption, pH, and molecular weight during degradation period. Mesenchymal stem cells (MSCs) were seeded and cultured in three-dimensional PLGA scaffolds to fabricate *in vitro* tissue engineering bone, which was investigated by cell morphology, cell number and deposition of mineralized matrix. The proliferation of seeded MSCs and their differentiated function were demonstrated by experimental results. To compare the reconstructive functions of different groups, mandibular defect repair of rabbit was made with PLGA/MSCs tissue engineering bone, control PLGA scaffold, and blank group without scaffold. Histopathologic methods were used to estimate the reconstructive functions. The result suggests that it is feasible to regenerate bone tissue *in vitro* using PLGA foams with pore size ranging from 100–250 μm as scaffolding for the transplantation of MSCs, and the PLGA/MSCs tissue engineering bone can greatly promote cell growth and have better healing functions for mandibular defect repair. The defect can be completely recuperated after 3 months with PLGA/MSCs tissue engineering bone, and the contrastive experiments show that the defects could not be repaired with blank PLGA scaffold. PLGA/MSCs tissue engineering bone has great potential as appropriate replacement for successful repair of bone defect. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 74A: 562–569, 2005

Key words: poly(D,L-lactide-*co*-glycolide); mesenchymal stem cells; bone tissue engineering; mandibular defect

INTRODUCTION

The requirements for new bone to replace or to restore the function of traumatized, damaged, or lost bone are a major clinical and socioeconomic need. Bone tissue engineering is a promising option for bone regeneration, and mesenchymal stem cells (MSCs) are a promising option for bone tissue engineering.[1–3] Some studies have demonstrated that tissue engineering scaffolds and growth factors can accelerate cell growth, bone formation, and osseous repair at the defect site.[4–6]

Porous biodegradable polymers are widely used in biomedical applications, especially tissue engineering for tissue repair and regeneration. Porous polymer scaffolds are used for both *in vitro* cell seeding and *in vivo* cell transplantation in tissue engineering studies.[7–11] For tissue engineering system, the scaffold provides a temporary support for cell growth and the cells grow within the polymeric scaffold. The polymeric scaffold degrades step by step with time and makes space for cell growth. As one of the most commonly used biodegradable polymers, PLGA is a desirable and very attractive polymer for fabricating porous scaffolds, because it biodegrades into lactic acid and glycolic acid by hydrolysis of ester bonds, which are removed from the body by normal metabolic pathways, relatively harmless to the cells and tissues.[12] At the same time, the degradation rate of PLGA can be controlled by varying copolymerization ratio, molecular weight, and polydispersity.[13] Porous PLGA foams have been utilized for the regeneration of various tissues and organs including bone[14] and liver.[15] The previous studies showed that PLGA foam could support marrow stromal osteoblast proliferation and differentiation both *in vitro* and *in vivo*.[16,17] The porous

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PLGA can be fabricated as a bone-like external geometry and allowed an individual site-specific insertion of implants during surgery. Osteoblasts coaxed with the PLGA material were viable under cell culture conditions and remained viable after implantation.[18]

For tissue engineering bone with porous scaffolds, more studies were focused on *in vivo* approaches. Because *in vitro* bone formation using three-dimensional biodegradable scaffolds was firstly reported by Ishaug et al.,[16] *in vitro* tissue engineering bone received more and more attention, and it is presumed to be more useful for a batch production and may allow more precise control of osteogenesis during the process than the *in vivo* approach.[19] Several types of bioreactor systems have been developed to enhance bone formation *in vitro*. Teral et al.[19] developed a new rotational oxygen-permeable bioreactor system (ROBS) that aimed at supplying optimal oxygen levels and continuous hydrostatic pressure to a tissue-engineered structure consisting of biodegradable polymer scaffold seeded with osteoblasts. All experimental results showed that *in vitro* bone formation was feasible; however, the level of bone formation is not enough. We hope to directly fabricate bone engineering organs or tissues by *in vitro* culture, just as tissue engineering skin, which also is the final aim, but now it could not be realized for tissue engineering bone *in vitro*. At present, it is a good approach that the seeded scaffold is cultured *in vitro* until adequate bone tissue formation, and then it is implanted into body to repair defect or form new tissue. Until now, though there are many studies about *in vitro* bone formation,[20–22] the real animal model experiments using *in vitro* tissue engineering bone to repair animal defect are few.

Animal models are often used to evaluate new ways of inducing or influencing bone growth. A frequently used animal model is repair of "critical size" defect. The "critical size" implies that the defect can not heal spontaneously. The rabbit mandibular defect model has been used in some studies, such as evaluation of ingrowth of bone substitutes, growth-stimulatory factors. To evaluate the treatment effect, bone growth inside the defect traditionally has been measured histologically using sections through the center of the defect, which is considered as the "golden standard."[23]

We propose to repair bone defects in a cell-scaffold-based tissue engineering approach using fully resorbable synthetic scaffold. For realizing the aim, the seeded porous PLGA scaffold was cultured *in vitro* until adequate tissue formation to attain tissue engineering bone, and then it was implanted into the mandibular defect site of rabbits. In this article, PLGA porous scaffolds were prepared, and its *in vitro* degradation behavior was investigated. MSCs were cultured in PLGA scaffolds to fabricate *in vitro* tissue engineering bone. The cell morphology, cell number, and deposition of mineralization were used to investigate bone formation. The mandibular defects of rabbits were repaired with PLGA/MSCs tissue engineering bone, control PLGA scaffold, and blank group without scaffold, respectively. The restoration status was followed with different time by histopathologic methods.

EXPERIMENTAL

The synthesis of PLGA (85/15)

D,L-lactide and glycolide were prepared by our lab. Stannous octoate, chloroform, and other solvent used were purchased from Shanghai Chemical Reagent factory.

D,L-lactide (17 g) and glycolide (3 g) were melted at 140°C for 30 min with stir under nitrogen atmosphere. Stannous octoate was added to the molten monomer and polymerized for 10 h at 150°C. After polymerization, PLGA was dissolved in chloroform, precipitated in excess methanol, and then filtered. The PLGA sample was vacuum-dried at 40°C for 24 h. The molecular weight (M_w) of the PLGA is 59,000 g/mol, determined by GPC.

Fabrication and characterization of scaffolds

The porous scaffolds were fabricated using an established solution-casting/salt-leaching technique with NaCl as the porogen.[1] Briefly, 1 g PLGA was dissolved in 10 ml chloroform, and then 8 g NaCl particles sieved to 250–350 μm was added. The suspension was cast in a 10-cm aluminum foil dish, air-dried for 48 h, and subsequently vacuum-dried for 12 h to remove remaining solvent. The aluminum foil was exfoliated after heated to 145°C for 2 min. The resulting PLGA/salt composite membranes were then immersed in distilled deionized water (DW) for 48 h (DW was changed every 6 h) with ultrasonic vibrations to leach out the salt. After being air-dried for 48 h and vacuum-dried for 12 h, the PLGA scaffolds were stored in a desiccator. Mercury intrusion porosimetry was used to determine the porosity of scaffold. The mercury filling pressure was applied up to 200 psia. The porous morphologies of the scaffolds were studied using scanning electron microscopy (SEM; Hitachi S-2360N). After being sputter-coated with gold, the polymer scaffolds were viewed by SEM under 15 kV.

In vitro degradation analysis

For *in vitro* degradation experiments, the PLGA scaffolds with about 90% porosity were cut into round

sample pieces with dimension of 7 × 4 mm (diameter × thickness). The samples were sterilized by UV exposure and placed into sterile vials containing 20 mL phosphate-buffered saline (PBS: 0.2 mol/L, pH 7.4 < 0.2). Then the sealed vials were stored at 37°C and allowed to degrade under slow agitation. For reducing difference, the samples were stored into PBS to begin degradation at different time. At the end of degradation experiments, all samples were taken out of the bottle, and the wet samples were weighed (Ww) after surface wiping. The pH of the medium for every sample was recorded. The dry samples (Wd) were weighed after being air-dried and vacuum-dried for 12 h, respectively. The weight average molecular weight of the scaffold PLGA polymer and degraded samples (M_w) were determined by GPC (Waters-150C). Tetrahydrofuran was used as an eluent (flow rate: 1 mL/min), and calibration was performed using polystyrene standards.

MSCs culture and *in vitro* bone formation

Seeding of scaffolds

Rabbit MSCs were obtained from the tibia of an immature (2 weeks old) White New Zealand rabbit as described by Solchaga et al.[24] The MSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Second-passage cells were used for the experiments.

The PLGA scaffolds (0.5 × 0.2 × 1.0 cm) were prewetted in culture medium [Dulbecco's modified Eagle's medium (DMEM), Gibco] for 12 h after being disinfected by ^{60}Co. The MSCs were suspended at the concentration of 1×10^6 cells/mL, and 0.1 mL of the cells suspension was poured onto each scaffold. The cells were allowed to adhere to the scaffolds for 3 h in an incubator, and then the scaffolds were covered with 2 mL of media (DMEM). Culture media were complete media supplemented and changed every three day.[1]

Cells morphology and cell numbers

After cultured for different days, the specimens were taken out of the culture media and rinsed twice and then 0.7 mL assimilation liquid (0.25% trypsin and 0.02% EDTA) was put into the specimens to assimilate for 3–5 min. After assimilation ended using 0.1 mL FBS, the liquid in the specimens was squeezed out and the cells were counted by cells count board. After colored by hematoxylin (HE), the cell morphology on scaffold was observed with different cultivation time by inverted phase contrast microscope (Leica model: DM IRB, Shanghai).

Calcium knur and tetracycline stain

After being cultured 21 days, the mineralized tissue was deposited below the scaffolds/MSCs surface, and Calcium knur was observed by inverted phase contrast microscope. The mineralized tissue also can be observed by fluorescence optical microscope (Olympus, PM-10ADS, BH-2, Japan) after tetracycline stain. Tetracycline, 10 μg/mL, was put into culture media. The specimens were taken out of the culture media and rinsed twice with PBS after continuing culture 24 h. The cultures were then frost chopped and observed by fluorescence optical microscope.

Animal model

A total of 12 mature White New Zealand rabbits (2.5 kg) were used. The rabbits were bred in different cages and held in a room at ambient temperature. Prior to operation, the rabbits were sedated by intravenous injection with pentobarbitol sodium. Then through-and-through osseous defects were created on mandibles with size 12 × 5 mm, which is so-called critical size defect. All rabbits were divided into three groups stochastically. In the experimental group, cell-scaffold-based tissue engineering bone and blank PLGA scaffolds were implanted into the mandibular defect site. In the control group, no any scaffold was implanted. All rabbits were euthanized after 6 or 12 weeks and the mandibles were harvested intact. The mandibles underwent macroscopic and histological analysis.

Histological evaluation

Histological specimens were fixed in 10% neutral buffered formalin for 7 days, followed by decalcification and dehydration, and finally embedded in paraffin. Sections of 5–6-μm thickness were cut at the cutting edge from one-half of the embedded specimen, and the samples were stained with HE.

RESULTS AND DISCUSSION

Morphology of the scaffolds

For tissue engineering scaffolds, the morphology (pore size, shape, and interconnectivity) is very important, which can affect cellular growth and tissue regeneration. If the pores are too small, the cells can not enter into scaffolds, and the nutrients and metabolites

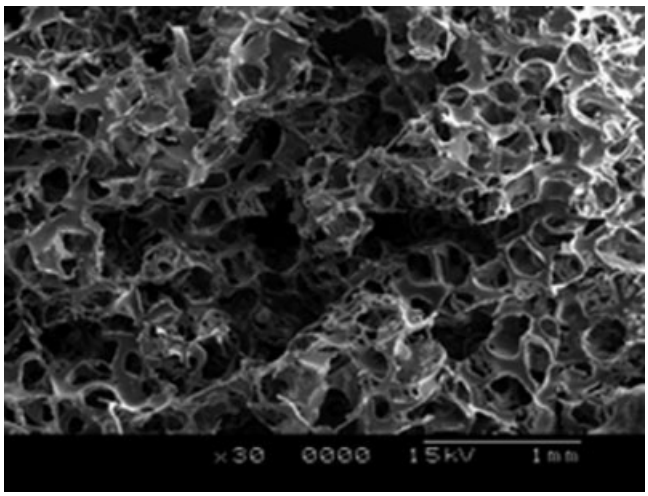


Figure 1. SEM micrographs of porous PLGA (85/15) scaffolds.

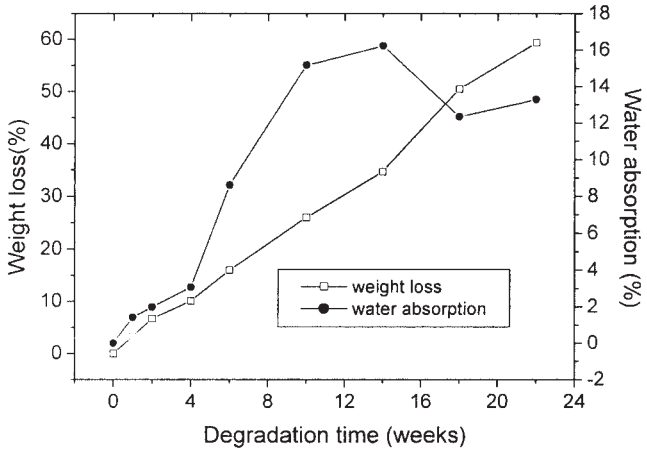


Figure 2. The change in weight loss, water absorption of PLGA scaffold with degradation time.

can not transit between matrix and environments; if the pores are too large, the cells easily move and can not fully adhere on the scaffolds. An ideal polymeric tissue engineering scaffolds should be highly porous to allow cell seeding and cell growth into the matrix and also to allow nutrients and wastes to permeate while preventing the migration of undesirable cells and tissues to the healing site.[11] At the same time, an open pore structure for porous scaffolds are often required in many tissue engineering application in order to maximize cell seeding, attachment, growth, extracellular matrix production, vascularization, and tissue ingrowth.[25] PLGA with M_w 59,000 was synthesized by copolymerization of monomer LA and GA with a 85:15 LA:GA molar ratio. Figure 1 shows porous structure of PLGA tissue engineering scaffolds fabricated by the solution-casting/salt-leaching method. It can be found that the scaffold had a highly porous structure. The pore size ranged from 100 to 250 μm, which was consistent with the size of NaCl particle. The pores were well interconnected throughout the scaffold matrix. Pore size, shape, and porosity of the scaffold were controlled primarily by the content and particle size of the porogen NaCl. With increasing salt weight fraction or salt particle size, the porosity and the median pore diameter of prepared scaffolds gradually increases, which is consistent with previous reports.[25] From our experimental results, it can be found that the distribution of interconnected pores and size of the scaffolds are regular and uniform with porogen over 85%.

In vitro degradation of the PLGA scaffold

Degradation properties of polymeric scaffold are of crucial importance for success of tissue engineering. The rate of degradation can affect many cellular processes including cell growth, tissue regeneration, and host response, so the rate of degradation must be in accordance with cell growth. As one of the most important biodegradable polymer for surgery, tissue engineering, and drug delivery, the degradation properties of PLGA have been studied by many authors.[25,26] Recently, Lu et al.[25] investigated the *in vitro* degradation of porous PLGA foam during a 20-week period in pH 7.4 PBS and their *in vivo* degradation after implantation in rat mesentery for up to 8 weeks. Holy et al.[26] also studied *in vitro* degradation behaviors of PLGA 75:15 foam in PBS at pH 5.0, pH 6.4, and pH 7.4. Some of their results are similar, and some are very different. Because many factors, including copolymerization ratio, molecular weight, polydispersity, specimen size and configuration, and environmental conditions affect the degradation process. So previous experimental results can only be considered as reference. For different PLGA scaffold, the degradation properties

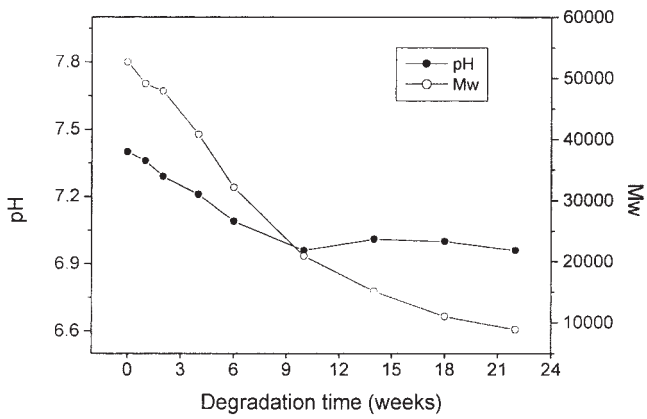


Figure 3. The pH and M_w change of PLGA scaffold with degradation time.

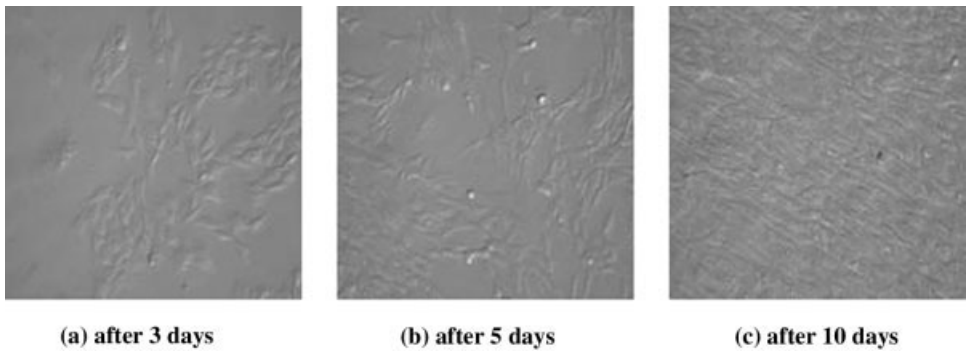


Figure 4. The cell adhesion and morphology on scaffold with different cultivation time by inverfted phase contrast microscope (original magnification ×10).

must be studied before it is used to culture cell and animal experiments.

The PLGA 85: 15 scaffolds with 90% porosity were characterized for *in vitro* degradation in PBS. The weight loss (WL) data for PLGA scaffolds under different hydrolyzing time are summarized in Figure 2. According to Figure 2, the WL increased with degradation time. The PLGA scaffolds lost mass at fast and constant rate, losing a total of 60% of their original mass in 22 weeks. The degradation rate was calculated of 2.7% per week. As shown in Figure 3, the weight-average molecular weight (M_w) of the PLGA scaffold decreased with hydrolyzing time, as determined by GPC. For the initial 10 weeks, the molecular weight of the sample decreased at approximately constant rate of 3170 g/mol/week. After 10 weeks, the rate of molecular weight decrease was gradually reduced. The pH variation patterns of the media containing the scaffold are shown in Figure 3. The figure shows that the pH of the media decreases at a constant rate until week 10 and then nearly does not change, which is in accord with molecular weight loss of PLGA.

Some of above results are different from Lu LC' reports.[25] According to their results, the weight of PLGA 85: 15 (M_w 142,800) foam with 90% porosity remained relatively constant throughout degradation process. The difference of molecular weight is main effect factor, and PLGA with low molecular weight resulted faster degradation.

Cell culture and *in vitro* bone formation

The MSCs seeded into PLGA scaffold continued to proliferate over the 20-day *in vitro* culture period in media. The cell adhesion and morphology on scaffold with different cultivation time was showed by inverted phase contrast microscope (Fig. 4). The MSCs began to attach to the pore surface of the scaffold and grow 6 h after seeded into the scaffold. But only a fraction of cells can attached to the polymer scaffold,

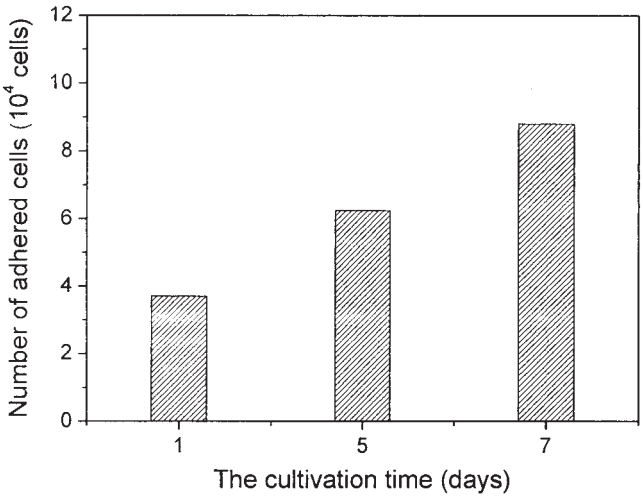


Figure 5. The numbers of MSCs attached in the PLGA scaffold with different times.

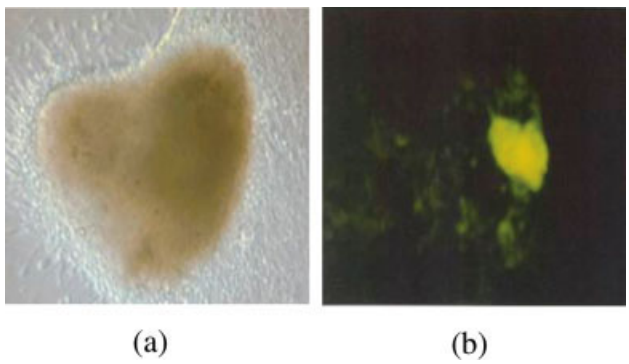
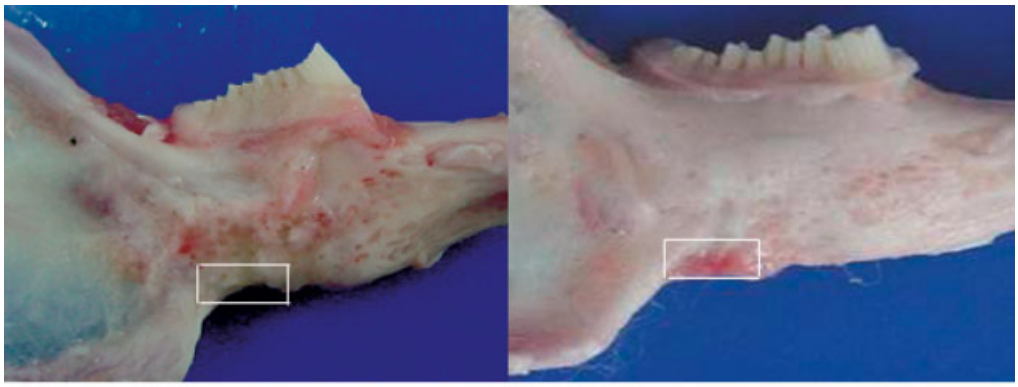


Figure 6. Calcium knur images after being cultured 3 weeks: (a) shown by inverted phase contrast microscope (original magnification ×400); (b) shown by fluorescence optical microscope after tetracycline stain (original magnification ×100). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



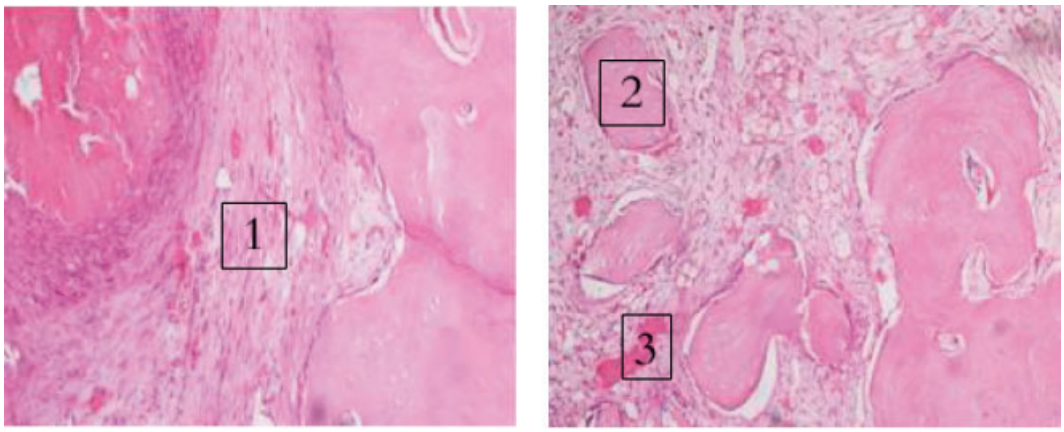
(a) (b)

Figure 7. The macroscopic analysis of bone defect repair: (a) the bone defect still retain after 12 weeks of healing for the blank group (without scaffold); (b) the defect has been filled with new bone for the PLGA/MSCs scaffolds group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and more MSCs exuded from the pore of scaffold. After 1 day's culture, about 3.7×10^4 cells, which was 37% of cells compared to initial seeding (10^5 cells), were attached to the scaffold. However, it was demonstrated that MSCs rapidly grew in the scaffold and more cells were attached to the pore surface from both results of microscope and number of cells. Two days later, cells began to congregate on some area. After 7 days' culture, the cell numbers reached 8.8×10^4 cells, and cells monolayer covered the pore surface (Fig. 5). After the medium was changed, the cells grew with faster speed and proliferated to form multilayers. The mineralization is a critical indicator of osteogenesis *in vitro* and osteogenesis differentiation of MSCs.[16,27–29] After MSCs were seeded into PLGA scaffolds, the cells were rapidly attached and grew. Some small granules of calcification can be seen within 2 weeks throughout the extracellular matrix by inverted phase contrast microscope, which is similar to that of previous studies.[19] The degree of mineralization was increased with time, and the calcium knur became larger after 3 weeks (Fig. 6).

Animal model

All animals recovered well after the surgical procedure, and no macroscopic signs of infection or other ailments were found during the healing period, which shows that the PLGA scaffold has good biocompatibility. After 6 weeks of healing, some polymer still



(a) (b)

Figure 8. Photomicrographs of histological sections 6 weeks after operation: (a) no sign of bone union and blood cells instead of much fiber connective tissues (1) for blank group (without scaffold; HE staining, original magnification ×200); (b) large quantity of bone cells and new formation bone tissue (2) with plenty of new formation blood vessel (3) for experiment group (with PLGA/MSCs) after implantation into rabbit for 6 weeks (HE staining, original magnification ×200). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

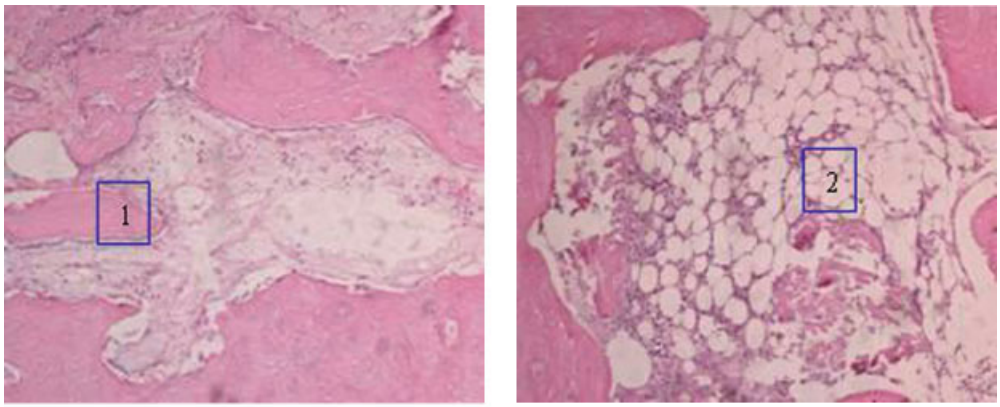


Figure 9. Photomicrographs of histological sections after scaffolds were implanted into rabbit for 12 weeks: (a) small amount of bone formation (1) at the rims for the PLGA scaffold experimental group (HE staining, original magnification ×400); (b) the defect totally filled with new bone for the PLGA/MSCs scaffolds experimental group, large quantity of mature fatty bone marrow (2) with adipose cells and blood cells in tissue (HE staining, original magnification ×200). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

existed in defect site. After 12 weeks, polymer was completely degraded and absorbed, and no remnant of polymer was visible in the connective tissue, which show faster rate of degradation *in vivo* than *in vitro.* Soon after implantation in the defect site, new bone formation took place at the rims of dependent and proceeds toward its center. Compared with the group without scaffolds in defect site, the groups with PLGA scaffold and PLGA/MSCs have a different degree to promote bone regeneration. In control group without scaffolds there was no any sign of bone union, and the surface of bone defect was covered with fibrillar configuration. The reason is that the bone regeneration is very slow, and fiber connective tissues grow fleetly and occupy bone healing space. After 12 weeks, the defect has been completely filled with new bone for the PLGA/MSCs scaffolds group, and there is large quantity of mature fatty bone marrow with adipose cells and blood cells [see Figs. 7(b), 8(b), and 9(b)]. For the group with PLGA scaffold, bone defect still remains, and only bone forms at the rims [Fig. 9(a)]. Tissue engineering porous materials can be as scaffolds of cell creeping, and a guide of bone regeneration. But control scaffolds without compound cells have no bone inducement function and can not be used to repair large area defect, which is proved by experimental results [Figs. 7(a) and 8(a)].

CONCLUSION

It can be concluded that porous PLGA scaffolds, pore size ranging from 100 to 250 μm, can be used to fabricate *in vitro* tissue engineering bone with MSCs, which can effectively repair mandibular defect of rabbits. The defect can be completely recuperated after 3 months with PLGA/MSCs tissue engineering bone, and the contrastive experiments show that the defects could not be repaired with blank PLGA scaffold. Tissue engineering using *in vitro* tissue engineering bone has demonstrated potential to offer an alternative and effective method for treating mandibular defects and other bone defects.

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