

Repair of critical size bone defects with porous poly(D,L-lactide)/nacre nanocomposite hollow scaffold

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ABSTRACT

الأهداف: عمل سقالة تقويمية جوفاء بمركب نانو عرق اللؤلؤ [porous poly (D,L-lactide) nacre].

الطريقة: أُجريت هذه الدراسة في قسم جراحة العمود الفقري، الجامعة الطبية الجنوبية، غوانغزو، الصين وذلك خلال الفترة من سبتمبر 2010م إلى سبتمبر 2011م. لقد قمنا بتحضير جزيئات نانو عرق اللؤلؤ باستخدام عملية فيزيائية، وقمنا بتحديدنا بواسطة انحراف الأشعة السينية وإرسال الفحص المجهر الإلكتروني وذلك من أجل عمل سقالة بواسطة معالجة ترشيح الأملاح. لقد قمنا باختبار شكل وتركيب جزيئات السقالة بواسطة الفحص المجهر الإلكتروني والمسح الضوئي واختبار الصفات الميكانيكية أيضاً. كما أننا قمنا بتقييم الصفات الحيوية بواسطة زراعة الخلايا في المختبر. شملت الدراسة 36 أرنب قمنا بتقسيمهم عشوائياً إلى 3 مجموعات. وقمنا بزراعة العيوب إما بالسقالة التقويمية الجوفاء أو السقالة التقويمية الجوفاء بمركب نانو عرق اللؤلؤ. وبعد ذلك قمنا بتحليل النتائج بتقييم صور الأشعة وكثافة معادن العظم وذلك من أجل مراقبة إعادة تقويم العظام.

النتائج: لقد كانت جزيئات نانو عرق اللؤلؤ كروية الشكل، وكان قطرها يتراوح ما بين 45-95، لقد تواجدت السقالة في تركيب مسامي مرتبط حيث كان حجم المسام 322.5 ± 50.8 ، كما أنه أظهر قدرة مسامية عالية ($82.5 \pm 0.8\%$)، بالإضافة إلى قوة ضغط جيدة (4.5 ± 0.25). وأظهرت اختبارات التوافق الحيوي الأساسية في المختبر بأن الخلايا قد كانت متلاصقة ومتكاثرة بشكل جيد في السقالة. وأشارت الدراسات التي أُجريت على الحيوانات بأن السقالة قد قامت بتقويم العيوب في أحجام العظام وذلك خلال 12 أسبوع.

خاتمة: أظهرت الدراسة أنه بالإمكان الاستعانة بطريقة السقالة هذه في القياسات الحيوية لهندسة الأنسجة العظمية.

Objectives: To generate a novel porous poly(D,L-lactide)/nacre nanocomposite hollow scaffold.

Methods: This study was performed in the Department of Spine Surgery, Southern Medical University, Guangzhou, China from September 2010 to September 2011. Nacre

nanoparticles were prepared using a physical process and identified by x-ray diffraction and transmission electron microscopy, to generate a novel scaffold though the salt leaching processing technique. The morphology and structure properties of this scaffold were further investigated under scanning electron microscope and mechanical property testing. Additionally, the biological characteristics were evaluated by cell culture experiments in vitro. Thirty-six rabbits were randomly divided into 3 groups. The defects were implanted with/without poly(D,L-lactide)/nacre scaffold or poly(D,L-lactide) scaffold. The results were assessed by radiographs and bone mineral density to monitor bone repairing.

Results: The nacre nanoparticles were spherical in shape, with a diameter range from 45-95 nm. The scaffolds possessed an interconnected porous structure with an average pore size of $322.5 \pm 50.8 \mu\text{m}$, and exhibited a high porosity ($82.5 \pm 0.8\%$), as well as good compressive strength of 4.5 ± 0.25 Mpa. Primary biocompatibility experiments in vitro showed that cells adhered and proliferated well on the scaffolds. The animal study further demonstrated that the scaffolds could repair the critical size segmental bone defects in 12 weeks.

Conclusion: Newly established scaffolds may serve as a promising biomaterial for bone tissue engineering.

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A bone defect is a major problem in the clinic, such as in trauma, and tumor resection. Autografts and allografts have been used to heal bone defects, but both treatments have limitations.^{1,2} Nowadays, bioactive materials have become more and more important in applications for bone regeneration.³ Recently, much attention has been paid to nacre, which is a novel bone substitute biomaterial.⁴ However, functional studies of nacre nanoparticles are very limited, especially the application of these particles for bone tissue engineering. Poly(D,L-lactide) (PDLLA) is a common polymer that has been widely used in tissue engineering due to its biodegradability, biocompatibility, and its ability to be dissolved in common solvents. However, PDLLA has a much lower modulus of elasticity than living bone, and shows no osteoconductivity.⁵ To improve the bioactivity and mechanical properties of the scaffolds, we designed a novel hollow scaffold by incorporating nacre nanoparticles into the PDLLA. Recent in vivo and in vitro studies have shown that nacre is biocompatible, biodegradable, and osteoconductive.^{6,9} The aim of this study was to fabricate a novel porous PDLLA/nacre nanocomposite hollow scaffold, characterize its physicochemical properties, and investigate in vitro cell response to gain insights into its potential use as scaffold in bone tissue engineering. We tested the scaffolds in a 15-mm critically sized segmental radial defect in a rabbit model.

Methods. This study was performed in the Department of Spine Surgery, Southern Medical University, Guangzhou, China from September 2010 to September 2011. Southern Medical University institutional ethical board approval was received to conduct this study. Nacre powder with an average particle size of 100 μm was purchased from Pearl Company of Guangdong Ocean University, Zhanjiang, China. The PDLLA, with an average molecular weight (Mw) of 210,000 Dalton, was purchased from Foryou Company, Huizhou, China. Other reagents were in analytical grade and used without further purification.

Preparation of nacre nanoparticles. Nacre nanoparticles were prepared using a physical process. A planetary ball mill (Pulversette 5, Fritsch GmbH, Germany) with agate balls (diameter, 10 mm) and

bowls (volume 500 ml) were used for producing nacre nanoparticles as previously reported.¹⁰

Characterization of nacre nanoparticles. The crystalline phase of the obtained nacre nanoparticles was analyzed with an x-ray diffraction (XRD) (Philips PW3040/60, Almelo, Netherlands). The morphology of the nacre nanoparticles was examined by a transmission electron microscope (TEM) (Philips CM200-FEG, Amsterdam, Netherlands).

Preparation of PDLLA/nacre nanocomposite hollow scaffolds. The scaffolds were prepared by solvent casting and salt leaching processing technique, which contains 30 wt% nacre nanoparticle and 70 wt% PDLLA. Briefly, 2.1 g PDLLA was dissolved in 15 ml dioxane to prepare a 14% (W/V) solution. Then, the solution with PDLLA was mixed with 0.9 g nacre nanoparticles and 12 g salt particles until they were well homogenized. After homogenization, the mixed solution was cast into a cylinder-shaped metal mold (Figure 1) and then frozen at -5°C for 12 hours. Subsequently, every cylindrical sample was removed from the mold and freeze-dried at -45°C for 48 hours. These samples were further rinsed with double distilled water for leaching the salt, and washed repeatedly until neutral pH was reached. At last, the samples were dried in a vacuum oven at 35°C for 24 hours and stored in a desiccator until characterization.

Characterization of scaffolds. The morphology of the newly generated PDLLA/nacre nanocomposite hollow scaffolds was analyzed by scanning electron microscope (SEM) (S-450, Hitachi, Tokyo, Japan). The average pore sizes were determined by measuring the size of 10 random pores from the SEM images. The porosity of the scaffolds was measured in distilled water by the Archimedes method.¹¹ The compressive strength

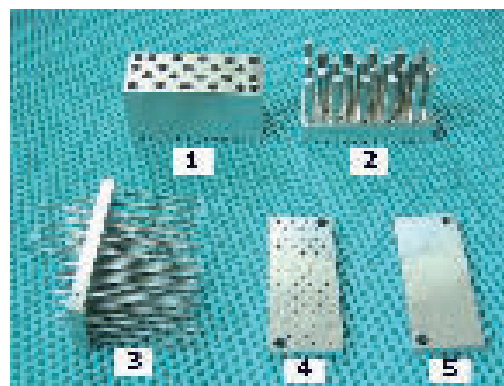


Figure 1 - Cylinder-shaped metal mold. 1. Subject of mold; 2. Thimble press plate; 3. Locating plate with needles; 4. Baffle plate with mini-holes; 5. Baffle plate.

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of the scaffolds was tested at room temperature using an MTS 858 machine (MTS Systems, St. Paul, MN, USA) with a constant displacement rate of 2 mm/min. Five individual samples were tested for each experiment (dimension of 8 mm external diameter, 2 mm internal diameter, and 40 mm length).

In vitro evaluation of scaffolds. We used 2, 3-week-old male Wistar rats in this study, and the experiment was carried out according to the Institutional and National Guide for the Care and Use of Laboratory Animals. The rats were anesthetized with ketamine (90 mg/kg) (Guangzhou Pharmaceutical Factory, Guangzhou, China) and xylazine (8 mg/kg) (Guangzhou Pharmaceutical Factory, Guangzhou, China). Fresh bone marrow was extracted from the femurs and tibiae of Wistar rat. The rat bone marrow stromal cells (BMSCs) were collected and cultured as described previously.¹² Cells were cultured and expanded in a low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Omaha, NE, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Omaha, NE, USA), and 0.1% penicillin/streptomycin (Gibco, Omaha, NE, USA). Culture media were replaced every 3 days. At 80-90% confluency, the cells were trypsinized with 0.25% trypsin/1 mM EDTA (Gibco, Omaha, NE, USA) and replated at a density of around 1×10^5 cells/ml. The BMSCs used in the experiments were at passage 3.

For in vitro studies, samples with the size of 8 mm in diameter and 2 mm in height were sterilized using ethylene oxide gas. A 0.1 ml aliquot of cell suspensions were seeded onto the top of prewetted scaffolds placed in 24-well plates. The medium in the cell-loaded scaffold culture plate was removed after being cultured for 2, 4, 6, and 8 days, and 0.2 ml MTT solution was added to each sample. Following 4 hours incubation at 37°C in an air atmosphere containing 5% CO₂, dimethyl sulfoxide (DMSO) was used to dissolve the blue formazan reaction product, and the optical densities (OD) were determined using an enzyme-linked immunosorbent assay plate reader (ELX800, Bio-Tek, Burlington, Vermont, USA) at 570 nm. As a control, a 24-well plate free of scaffolds was also used as a negative control. Five replicate samples were tested for each condition (n=5). At predetermined time points of 3 days, the cell seeded scaffolds (n=3) were taken out of the culture and gently washed with PBS, and the cell morphology on the scaffolds was observed by SEM after fixing the samples with glutaraldehyde, dehydrating them with a graded series of ethanol, and treating with hexamethyldisilazane.

In vivo studies of repair of rabbit radial critical size bone defect. Thirty-six adolescent New Zealand white

rabbits from both genders weighing 2.0-2.5 kg were used. The experiment was carried out according to the Institutional and National Guide for the Care and Use of Laboratory Animals. Rabbits were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride (Guangzhou Pharmaceutical Factory, Guangzhou, China) and 5 mg/kg xylazine (Guangzhou Pharmaceutical Factory, Guangzhou, China). A 15-mm segmental osteoperiosteal defect was created in the radius bilaterally. For experimental groups, the rabbits with radial defects were implanted with PDLA/nacre nanocomposite hollow scaffolds, and control groups were implanted with PDLA hollow scaffolds. Rabbits implanted without any scaffolds were used as a blank control. At 4, 8, and 12 weeks postoperatively, 4 rabbits from each group were euthanized. Before being euthanized, animals underwent x-ray examination at 4, 8, and 12 weeks. Bone mineral density (BMD) of the radius repaired for 12 weeks in all 3 groups was measured by dual energy x-ray absorptiometry (DEXA) (XR-36, Norland, Connecticut, USA).

Statistical analysis. The quantitative data were expressed as mean \pm standard deviation (SD). Data were analyzed statistically using the Statistical Package for Social Sciences version 13.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using one-way ANOVA. A *p*-value less than 0.05 was considered statistically significant.

Results. Characteristics of nacre nanoparticles.

The diffractogram from XRD analysis of the nacre nanoparticles is shown in Figure 2a. The straight baseline and the sharp peaks in this diffractogram indicate that the samples were phase-pure and well crystallized. Transmission electron micrographs of the nacre nanoparticles were shown in Figure 2b. The nano-sized nacre crystals were spherical in shape, with a diameter range from 45-95 nm.

Characterization of the hollow scaffolds. Figure 3a showed that the novel scaffolds consisted of a cylindrical hollow structure. The scanning electron microscopy (SEM) images showed that the scaffolds were 3-dimensional porous structures, with good interconnections between the pores (Figures 3b and 3c). The average pore size of the scaffolds was 322.5 ± 50.8 μ m, and exhibited a high porosity ($82.5 \pm 0.8\%$), as well as good compressive strength of 4.5 ± 0.25 Mpa.

The BMSCs growth on the scaffold. The 3-(4,5)-dimethylthiazolium(-z-y1)-3,5-diphenyltetrazoliumromide (MTT) results (Figure 4) showed that the PDLA/nacre nanocomposite hollow scaffolds did not interfere with BMSC proliferation.

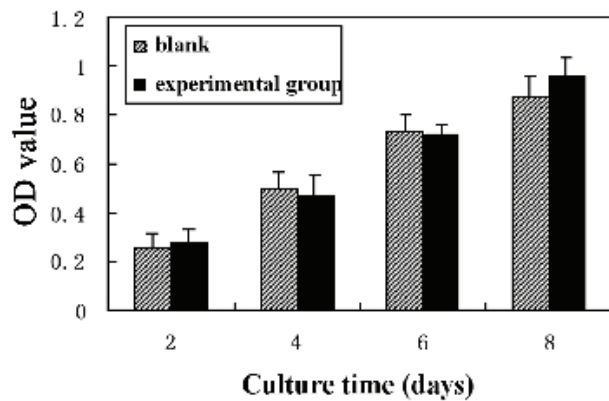
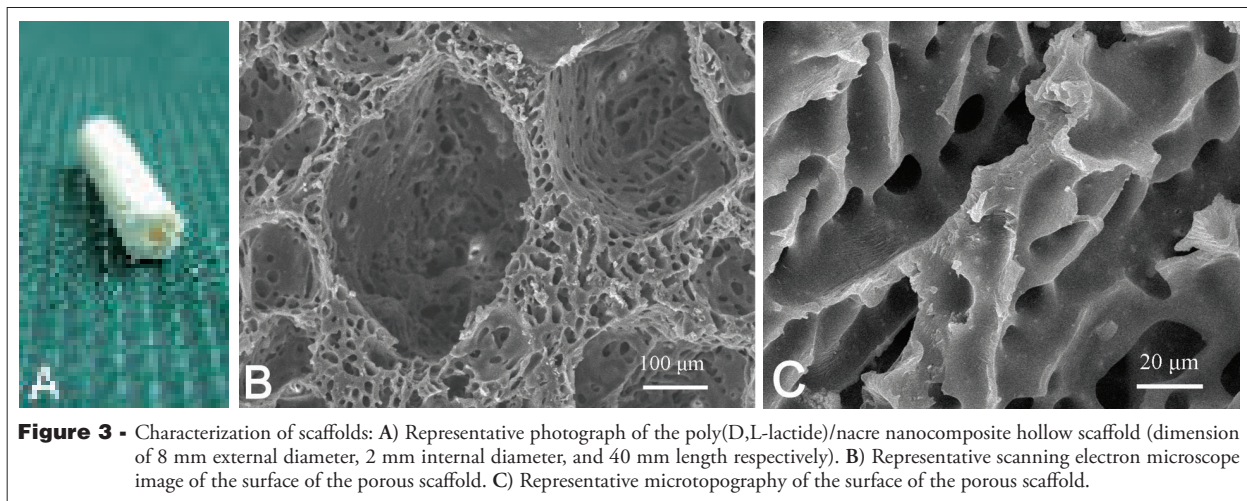
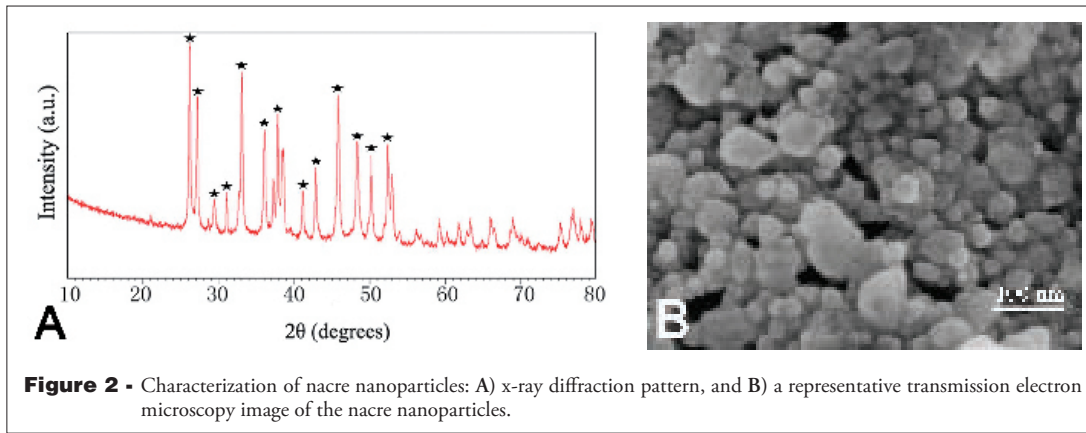
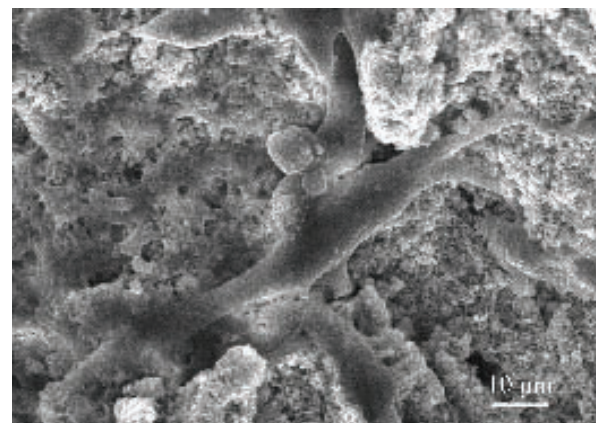


Figure 4 - Proliferation of cells responding to scaffold at various time points: MTT analysis. MTT - 3-(4,5)-dimethylthiazazo(-z-yl)-3,5-di-phenyltetrazoliumromide



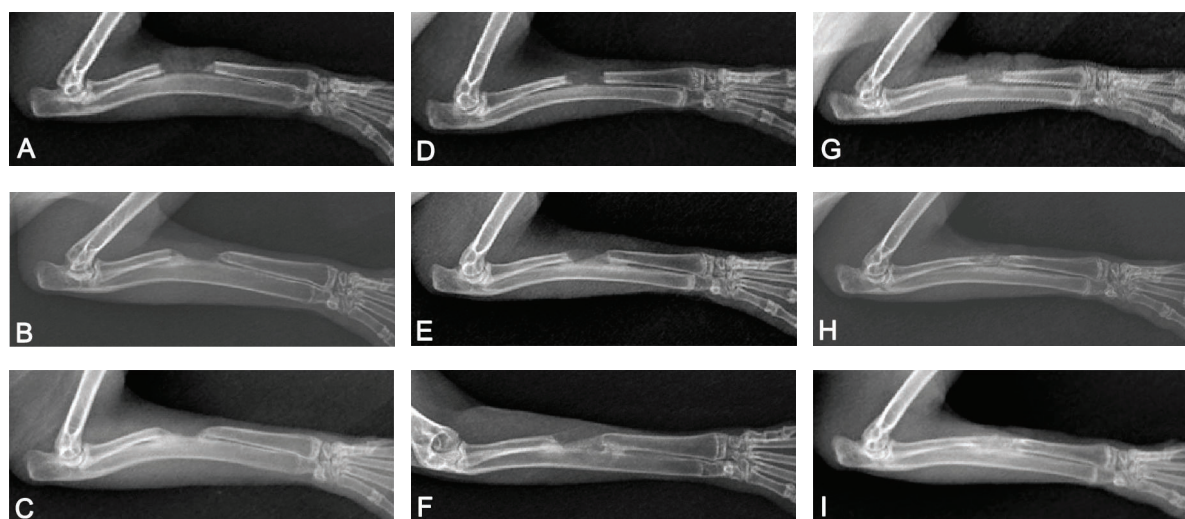


Figure 6 - X-ray microradiographs of the defect areas of rabbit radius at weeks 4, 8, and 12 after operation showing: A-C) Bone defect with no implantation; D-F) bone defect with implantation of poly(D,L-lactide) scaffold; G-I) poly(D,L-lactide)/nacre nanocomposite hollow scaffold repaired the defect.

Table 1 - Bone mineral density (BMD) of the repaired rabbit radius at 12 weeks with various scaffolds.

Scaffold group	No.	BMD (g/cm ²)	P-value*
Poly(D,L-lactide)/nacre scaffold	4	0.2368±0.016	0.075
Poly(D,L-lactide) scaffold	4	0.1476±0.017	0.000
Control	4	0.0615±0.015	0.000
Normal	4	0.2659±0.011	

Data are expressed as mean±SD, *versus normal

The MTT activity of the BMSCs increased with culture time. After 8 days of incubation, there was no significant difference between the experimental group and controls ($p=0.847$). The SEM images taken on the third day are shown in Figure 5. The BMSCs were observed to spread well, with an intimate contact with the surface of the scaffolds.

Repair of critical size rabbit radial bone defect.

Figure 6 shows x-ray microradiographs of the defect areas of rabbit radius at weeks 4, 8, and 12 after operation. In the experimental groups, a little callus formed after 4 weeks (Figure 6g). The margins and the bone tissue became cloudy along with the implanted scaffold after 8 weeks (Figure 6h). At 12 weeks, the medullary cavity achieved full recanalization, and bone defects were repaired completely (Figure 6i). The BMD results (Table 1) showed that the experimental group was similar to that of the normal specimens.

Discussion. An ideal bone scaffold should be biocompatible with osteoconductive and osteoinductive properties. Nacre is a promising natural bioceramic, which is biocompatible, and shows osteogenic activity.^{7,8} Recently, many studies have shown that nanometer-scale materials can provide better microenvironments for cell attachment and organization than their micrometer-scale counterparts,^{13,14} which lead us to use nano-sized nacre particles to fabricate PDLA/nacre nanocomposite hollow scaffolds for bone tissue engineering.

The geometry of the scaffold plays a crucial role in supporting osteogenic cell migration and survival in the scaffold.¹⁵ Figure 3a showed that the novel scaffolds consisted of a cylindrical hollow structure, which is beneficial to seed biological cells on the 3-dimensional scaffolds, and sufficient to leach the salt. The SEM images showed that the scaffolds were 3-dimensional porous structures, with good interconnections between the pores (Figures 3b and 3c). The average pore size of the scaffolds was $322.5\pm 50.8\ \mu\text{m}$, which is suitable for osteoblast seeding and growth.^{16,17} In addition, the scaffold in our study exhibited a relative high porosity ($82.5\pm 0.8\%$). High porosity is an important parameter for cell migration, growth, and flow transport of nutrients and metabolic waste.¹⁶ A well-generated scaffold should have not only appropriate porosity, but also satisfactory mechanical properties, which helps its implantation into the body.¹⁸ To obtain a scaffold with better mechanical strength, we generated the PDLA/

nacre nanocomposite scaffold, which has a compressive strength of 4.5 ± 0.25 Mpa, which is in the range of cancellous bone (2-10 MPa).¹⁹

It is well known that cell biocompatibility is the most important parameter for a scaffold in the bone tissue engineering field. The BMSCs were observed to attach well onto the strut surfaces of the scaffolds, which demonstrated that PDLLA/nacre nanocomposite hollow scaffolds have good biocompatibility to support cell adhesion. Furthermore, the cell proliferation assay showed that BMSCs grew normally with the scaffolds and these scaffolds were non-toxic for BMSCs. This study further shows that the application of PDLLA/nacre nanocomposite hollow scaffold to be a biomaterial for bone tissue engineering.

The results of in vitro experiments prompted us to investigate the properties of the PDLLA/nacre nanocomposite hollow scaffold in vivo. We used a critical size bone defect of rabbit radial to study the bone repair, as this type of defect is unable to heal by itself.²⁰ Thus, we could evaluate the action of each of the PDLLA/nacre nanocomposite hollow scaffolds in the bone repair. The PDLLA/nacre nanocomposite hollow scaffold had an excellent bone regeneration effect, as the critically sized (15 mm) segmental defect could be completely repaired, as shown by x-ray and BMD assay within 12 weeks. During the experiment, all rabbits remained in good health and did not show any wound complications, indicating the good biocompatibility of the implanted hollow scaffolds. In addition, the BMD results (Table 1) of the experimental group were similar to the normal specimens, and the BMD was significantly greater in the experimental group compared to the control group.

Nacre is the inner layer of the shell of the mollusk pearl oyster or bivalve. It is a hard tissue consisting of more than 95% calcium carbonate crystals, and around 5% of organic matrix.^{21,22} Nacre is a biomaterial that stimulates bone regeneration, while the nacre particles slowly dissolve away.²³ In this study, nacre nanoparticles were prepared by using a planetary ball mill, and the nano-sized nacre crystals were spherical shape, with a diameter range from 45-95 nm. Further studies should be performed to investigate whether nacre nanoparticles are more easily degradable, and to determine the degradation of the novel scaffolds for tissue engineering applications.

In conclusion, a novel PDLLA/nacre nanocomposite porous scaffold was developed by a solvent casting and leaching technique. Based on the above analyses and discussion, it can be concluded that the scaffold possessed an interconnected porous structure with an average pore

size of 322.5 μm , and the compressive strength reached 5.0 Mpa with a porosity of 82.5%, which would meet the basic requirements of growth for new bone tissue. In addition, the BMSCs cells attached and proliferated well on the scaffold during the cell culture period in vitro. The bone defect was successfully repaired over 12 weeks. In summary, this scaffold is a very promising biomaterial for bone tissue engineering.

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