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Histological study of surface modified three dimensional poly (D, L-lactic acid) scaffolds with chitosan in vivo

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Abstract Biocompatibility and tissue regenerating capacity are essential for biomaterials that used in tissue engineering. The aim of this study was to histologically assess the tissue reactions and bone conductivities of surface modified three dimensional (3-D) poly (D, L-lactic acid) (PDLLA) scaffolds, which were coated with chitosan via a physical entrapment method. The native PDLLA scaffold was prepared via thermally induced phrase separation technique and was characterized by scanning electron microscopy (SEM) and differential scanning calorimetry (DSC). Osteocalcin assay, a method to evaluate the bone formation potential, has shown that the osteocalcin production in chitosan-modified 3-D PDLLA scaffold group was significantly higher ($p < 0.05$) than that of in control. The tissue reactions and bone conductivities between surface modified PDLLA and native PDLLA scaffolds were evaluated using a rabbit radialis defect model in vivo and compared at different implantation intervals (2, 4, 8 and 12 weeks). The histological results have shown a higher bone formation potential and better biocompatibility of chitosan-modified 3-D PDLLA scaffolds as compared with the control group scaffolds.

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Introduction

Tissue engineering has emerged as a promising approach to regenerate and/or repair damaged organs as well as tissues. The approach is based on the use of a scaffold with special architecture as a template to reinforce and organize the regeneration of tissues [1–3]. The scaffolds inevitably come into contact with the cells and surrounding tissues. Thus, biocompatibility and tissue regenerating capacity are essential for the scaffolds that used in tissue engineering.

Poly (α-hydroxyl ester)s are currently being widely investigated as materials for regeneration of several tissues [4, 5], mainly because of their good biodegradable properties. However, due to the hydrophobic nature, the compatibility of pure poly (α-hydroxyl ester) scaffolds is still remaining a problem in tissue engineering field. Therefore, surface modification is performed on the poly (α-hydroxyl ester) scaffolds to improved their biological performance. With successful surface modification, the mechanical properties and functionality of a scaffold would be unchanged, but its biocompatibility and tissue regenerating ability would be improved [6].

Many methods have been performed for the surface modification of poly (α-hydroxyl ester) scaffolds . Various biologically active molecules were chemically or physically immobilized on poly (α-hydroxyl ester) matrixes [7, 8] in order to improve their biological performance. A previous study [9] have investigated the influences of both chemical and physical treatments of poly (D, L-lactic acid) with silk fibroin on the osteoblast growth behaviors in vitro. The results indicated that different surface treatments of poly (D, L-lactic acid) films had distinct effects on the osteoblast growth. The physical entrapment was superior to the chemical method in the improvement of

biocompatibility of poly (D, L-lactic acid) films. Chitosan is an amino polysaccharide which is derived from arthropod exoskeletons. Several studies [10–13] have investigated the effect of chitosan on cell growth and potential application in tissue engineering.. In our previous study [14], chitosan was successfully employed to surface modify poly (D, L-lactic acid) films. The results showed that surface modified poly (D, L-lactic acid) films had positive effects on the proliferation and differentiation function of rat calvaria osteoblast as compared with native poly (D, L-lactic acid) films.

The aim of this study was to employ chitosan to surface modify three dimensional (3-D) PDLLA scaffolds via a physical entrapment method. A rabbit radialis defect model was utilized to investigate its tissue reactions in vivo. Osteocalcin assay and histological observations were performed to evaluate the tissue reactions and bone formation potential of chitosan-modified PDLLA scaffolds as compared with native PDLLA ones.

Materials and methods

Materials

Poly (D, L-lactic acid) was provided by Chengdu Institute of Organic Chemistry, Chinese Academy of Science, with a weight average molecular weight (Mw) of 2.5×10^5. Chitosan was provided by Qingdao Medicine Institute of China. The degree of deactylation is about 85%. The viscosity-average molecular weight of the chitosan is about 8.8×10^5 determined by the Mark-Houwink equation. All osteocalcin assay agents were acquired from China Military Hospital.

Scaffold preparation [15]

To prepare viscous PDLLA solution, 2.0 g PDLLA sample was added to 30 mL of methylene chloride with magnetic stirring. 10 g of ammonium bicarbonate (particle size is around 100–300 μm) was then added to the PDLLA solution and mixed vigorously. A glass mold with a dimension of 3.5 mm diameter and 10 mm length was used in the present study. Polymer/salt/solvent mixture paste was casted into the glass mold and air-dried under atmospheric pressure to remove methylene chloride. After methylene chloride was evaporated, the semi-solidified samples were immersed into excessive amount of hot water (60 °C) until no gas bubbles were generated (≈45 min). The treated samples were placed into cold water for 15 min and then freeze-dried for 2 days and stored at –20 °C until use.

Surface modification of scaffold

Immobilization of chitosan on 3-D PDLLA scaffolds was performed according to Ref. [16] via an entrapment method. Briefly, PDLLA scaffolds were immersed into a mixture of acetone/chitosan solution (2%wt) =1:1 for 6 h. Then, the samples were rinsed six times with distilled water and phosphate buffered saline (PBS), respectively. Afterwards, the surface-modified scaffolds were freeze-dried for several days. All samples were sterilized using $Co^{60}\gamma$ irradiation with 2.0 Mrad dose.

SEM observation

Scanning electron microscopy (SEM) was employed to evaluate (a) surface/cross-sectional pore morphology of freeze-dried scaffolds, and (b) surface pore morphology of modified scaffolds with chitosan. Samples were freeze-dried and then gold-sputtered in vacuum and examined by SEM observation using XL30 scanning electron microscope (USA). The accelerating voltage used in this study was 15.0 Kv.

Porosity evaluation of PDLLA scaffolds

Porosity evaluation was performed according to the apparent densities method in previous study [17]. PDLLA scaffolds were freeze dried for 3 days and then kept in a desiccator at room temperature. The weight of dry samples, apparent weight of saturated samples and weight of wet samples were measured to an accuracy of 10^{-3} g (n = 6). The open porosity *Po* was calculated according to

$$Po = (m_3 - m_1)/(m_3 - m_2) \times 100\% \quad (1)$$

where: m_1, m_2 and m_3 are the mass of dry samples, apparent mass of saturated samples and mass of wet samples, respectively. For pore size and distribution characterization, 100 pores were measured for each sample.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC, Perkin–Elmer) was employed to investigate the thermal properties of scaffolds. Glass temperature (Tg) was measured in the first heating scan and the melting temperature (Tm) and its corresponding enthalpy (ΔHm) were determined in the second scan. Temperature was scanned from –40 °C to 200 °C with a heating speed of 10 °C/min.

Osteocalcin assay

Blood samples were acquired from rabbits just before killing at determined time period. After extracting the

serum from blood samples, they were placed in individual polypropylene tubes and frozen (–80 °C) until the end of the assaying. The procedure is according to Ref. [18] via a gamma irradiation counter (Gensys Laboratory Technologies, Elburn, IL). A standard curve for gamma counts was constructed by using parallel samples counting known concentration of osteocalcin.

Animal experiments

Thirty-two mature Japanese white rabbits weighting around 2–2.5 kg, underwent surgery on both radialises. Anteromedial arthrotomy was performed under intravenous pentobarbital with sodium anesthesia (20 mg/kg body weight). The radialis was exposed. A full defect was generated using a saw. The size of the defect was about 10 mm in length at the medium section of the whole radialis (Fig. 1). The defect was tightly filled with the sterilized 3-D scaffolds. Muscle and skin were sutured as separate layers.

The rabbits were killed 2–12 weeks postoperatively by an overdose of pentobarbital sodium. Eight rabbits killed at each time point of 2, 4, 8 and 12 weeks, respectively. For light microscopic observation, the samples were fixed in 10% neutral formalin, decalcified in Plank-Richlo solution at 4 °C, dehydrated and embedded in paraffin. A minimum of 10 saggital sections 6 μm thickness were prepared from the center of each defect so that the error due to sampling could be minimized. The samples were then stained with hematoxylin and eosin (H&E) as well as Masson for determining the structural details, cell appearance and bone formation.

Statistical analysis

Experiments were run in quadruplicate per sample. All data were expressed as means ± standard deviation (SD) for n = 4. Single factor analysis of variance (ANOVA) technique was used to assess statistical significance of results. Scheffe's method was employed for multiple comparison tests at level of 95% and 99%.

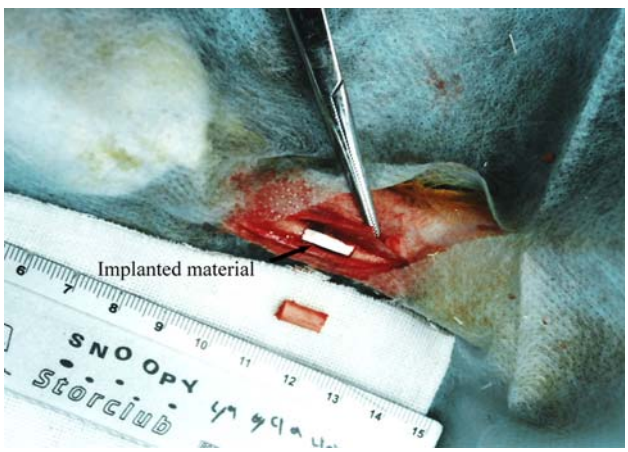

Fig. 1 Surgical pictures of rabbit radialis defect model

Results

Scaffold characterization

In this study, the poly (D, L-lactic acid) scaffold was prepared by a salt-leaching method. Poly (D, L-lactic acid) was first dissolved into methylene chloride, and the polymer solution was then mixed with ammonium bicarbonate microparticles in an appropriate proportion to form a malleable paste, which could be applied into molds with various shapes and sizes. Figure 2 shows SEM images of both surface-modified PDLLA and native PDLLA scaffolds. Surface and cross section morphologies of native PDLLA scaffold are shown in Fig. 2a and b, respectively. They display similar morphology characteristics composed of interconnecting pores with the mean diameter with average pore size of 142 ± 24 μm. Figure 2c displays the SEM image of surface morphology of chitosan-modified PDLLA scaffold. Compared to the surface morphology of native PDLLA scaffold (Fig. 2a), the chitosan modified scaffold shows no much difference in surface morphology. The average pore size was little decrease to 125 ± 27 μm for chitosan-modified one. The pore distribution evaluation displays that the percentage of pores located in 50–100 μm range are increased from 15% to 28% corresponding to that of decrease within 150–200 μm and 200–250 μm range for chitosan-modified PDLLA scaffolds, when compared to the control PDLLA scaffolds (Table 1). This is related to the physical shrinkage of entrapment treatment which used in the present study for surface modification purpose, thus leading to the decrease of pore size. This phenomenon was observed in our previous study [19], which employed silk fibroin to surface modifies PDLLA via entrapment method. Little difference in pore distribution located within 100–150 μm was observed between chitosan-modified scaffolds and native ones. A previous study [20] has revealed that the pore size and morphology of a scaffold strongly influenced cell adhesion behavior. However, no statistical difference on mean pore sizes between PDLLA scaffolds and chitosan-modified one was observed. Thus, the similarity of surface morphology between chitosan modified PDLLA and native PDLLA scaffolds is one of the most important results in this study.

Differential scanning calorimetry is a technique that can measure the thermal properties of a polymer. In the present study, it was employed to determine whether any change in polymer structure occurred during the scaffold fabrication

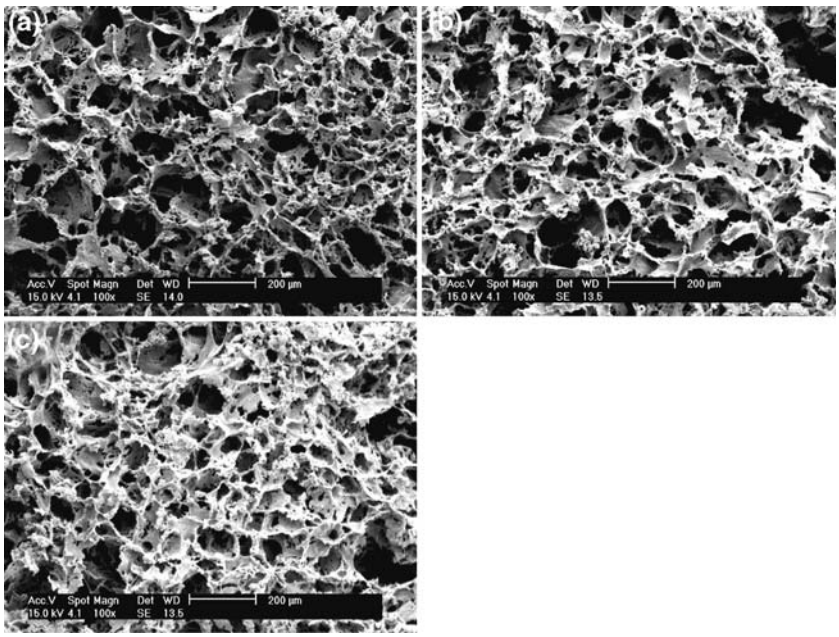

Fig. 2 SEM images of PDLLA scaffold and chitosan-modified one: (**a**) surface; (**b**) cross-section; and (**c**) surface morphology of chitosan-modified scaffold

Table 1 Porous characteristics of PDLLA scaffold and surface treated one with chitosan

Materials	Porosity (%)	Pore size (μm)	Pore distribution (%)			
			50–100 (μm)	100–150 (μm)	150–200 (μm)	>200 (μm)
PDLLA	91.2 ± 1.1	142 ± 24	15	48	28	9
Chitosan-modified PDLLA	89.4 ± 0.8	125 ± 27	28	53	14	5

process. Figure 3 shows the thermal properties of both native PDLLA and chitosan-modified PDLLA scaffolds. Glass transition temperature around 39.8 °C was observed for the native PDLLA scaffold. For the chitosan-modified PDLLA scaffold, a higher glass transition temperature was observed. This difference in Tg value between the chitosan surface-modified and native PDDLA scaffolds, may be ascribed to the physical entrapment of chitosan molecules onto the scaffold surfaces, which possess rigid molecular structure. Nevertheless, both raw PDLLA and chitosan modified PDLLA scaffolds show the similar crystalline melting temperature around 178 °C and melting enthalpy (ΔH_m) about 28.5 J/g. This data reflected that little structure change of PDLLA scaffold was happened after chitosan surface modification process. As Ratner [6] mentioned that an ideal surface modification, the bulk properties of materials would be unaffected and the biological performance of material would be improved. This result reflected that the surface modification using chitosan on PDLLA scaffolds was successful.

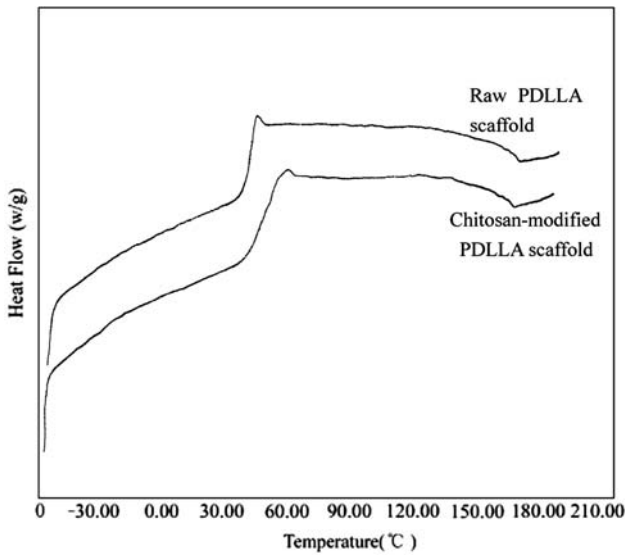

Fig. 3 DSC pictures of (**a**) PDLLA scaffold and (**b**) chitosan-modified PDLLA scaffold

Osteocalcin production assessment

Table 2 shows the relative osteocalcin production between the chitosan surface-modified and native PDLLA scaffolds. At time intervals of 2 and 4 weeks, the osteocalcin production in chitosan surface-modified scaffolds is

Table 2 The content of osteocalcin in serum of rabbit implanted with PDLLA scaffold and chitosan-modified scaffolds (ng/mL) (n = 4, mean ± SD)

Time (Weeks)	Samples	
	PDLLA scaffold	Chitosan-modified PDLLA scaffold
2	5.88 ± 0.56	6.94 ± 0.15*
4	3.11 ± 0.19	4.16 ± 0.42*
8	2.71 ± 0.61	2.18 ± 0.51
12	2.48 ± 0.31	2.99 ± 0.58

*$p < 0.05$ (compared to control)

statistically significantly higher ($p < 0.05$) than that in native PDDLA scaffolds. However, no significant difference between the chitosan surface-modified scaffold groups and the control group was observed at the time intervals of 8 and 12 weeks, respectively. The result suggests that chitosan surface-modification was beneficial to the osteocalcin production at the initial implantation stage. Osteocalcin, γ-carboxyglutamic acid protein, one kind of non-collagen protein in bone tissue, is a multi peptide substance which is produced by osteoblasts. It is one of the components to form bone matrix [21]. It is also one of the markers for osteoblast differentiation function. This above result reveals that the surface modification with chitosan is helpful for the gene expression of osteoblast differentiation, thereby enhancing the formation of new bone.

Histological observation

Two weeks

In the control group (n = 4), there was slight inflammatory reaction (Fig. 4a) with a few lymphocytes and macrophages observed on the scaffolds. Fibrous structure (FS) which is located at the interface between the surrounding bone and the implanted scaffold was clearly observed. This experimental result indicates the active fibroblast growth at the scaffold interface. Further, no osteoid matrix formation inside the implanted scaffolds was observed. In the chitosan surface-modified group (n = 4), both osteoid matrix formation and active osteoblast inside the scaffolds (Fig. 4b) were observed. Light inflammatory reaction also took place on the scaffolds, but with no FSs observed. This result indicates a good interface between the surrounding bone and the chitosan surface-modified scaffolds. From the above comparison, it is seen that the surface-modification of scaffolds using chitosan had a positive effect on tissue response at the initial stage of implantation.

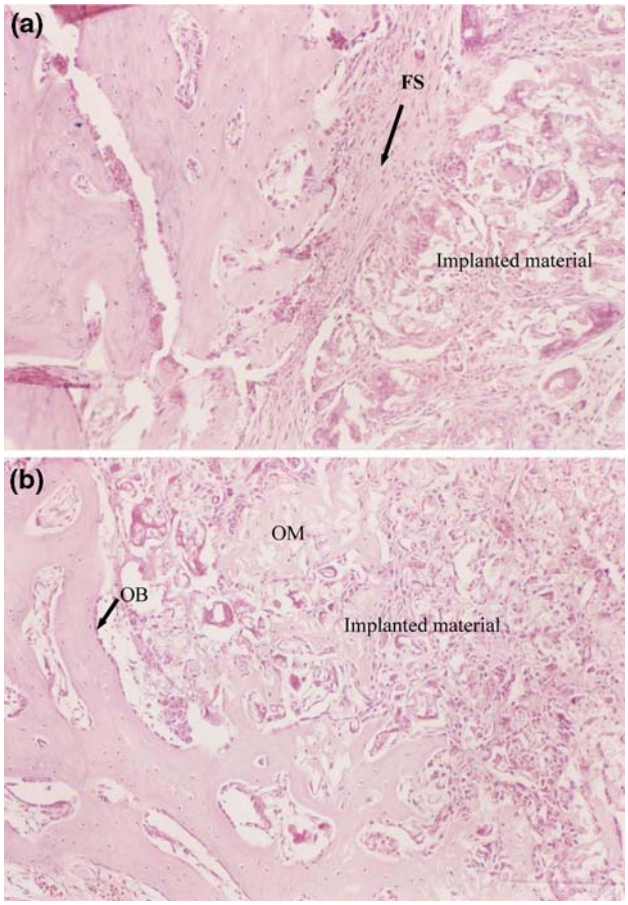

Fig. 4 Histological appearances of rabbit radialis defect implanted with PDLLA and modified PDLLA scaffolds after 2 week (H&E 100×): (**a**) PDLLA scaffold, fibrous structure (FS) formed at the interface between original bone and implanted material; and (**b**) chitosan-modified PDLLA scaffold. Osteoid matrix (OM) formed and osteoblast (OB) appeared inside the materials

Four weeks

Figure 5a shows the native (control) PDLLA scaffold implanted in rabbit radialis defect after implantation for 4 weeks. There was still no osteoid matrix formed inside the control scaffold. Meanwhile, the FS between the original bone and the implanted scaffold still could be observed and even became thicker than that after implantation for 2 weeks (Fig. 4a). Although many active osteoblasts were noticed in the area surrounding the native PDLLA scaffold, the produced FS prevented the migration of the osteoblasts into the interior of the scaffolds. Obvious degradation of the native scaffolds was observed at this time of implantation. In chitosan surface modified group, a large amount of osteoid matrix formation was found (Fig. 5b) as compared with that after implantation for 2 weeks (Fig. 4b). Some small bubble-like structures were present inside the implanted scaffolds, due to the degradation of the scaffold polymer. It worth noting that at this

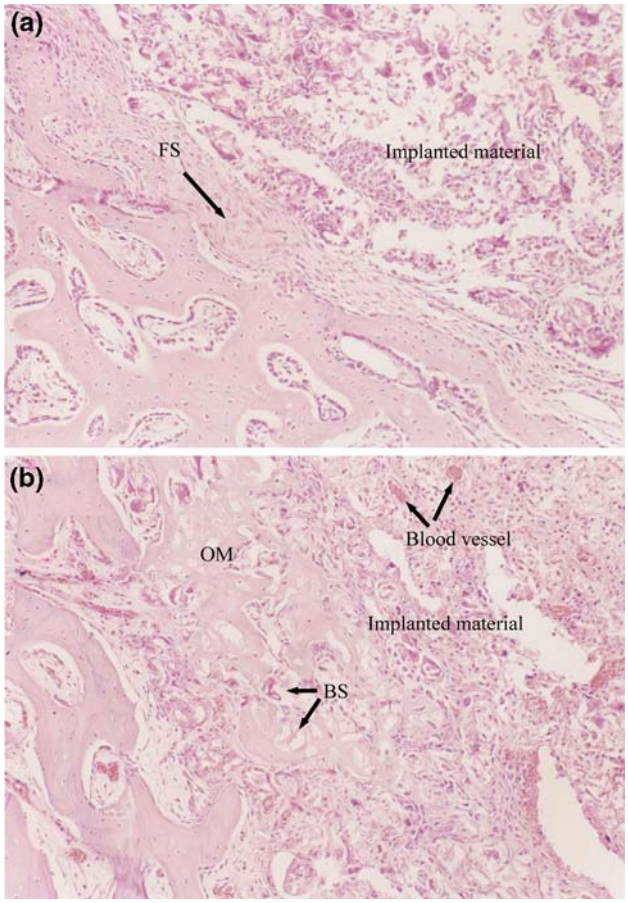

Fig. 5 Histological observations of rabbit radialis defect implanted with PDLLA and chitosan-modified PDLLA scaffolds after 4 week (H&E 100×): (**a**) PDLLA scaffold, fibrous structure (FS) still existed and no osteoid matrix formed inside implanted materials; and (**b**) chitosan-modified PDLLA scaffold, large amount of osteoid matrix (OM) formed inside the implanted materials and bubble-like structure (BS) were present inside implanted materials

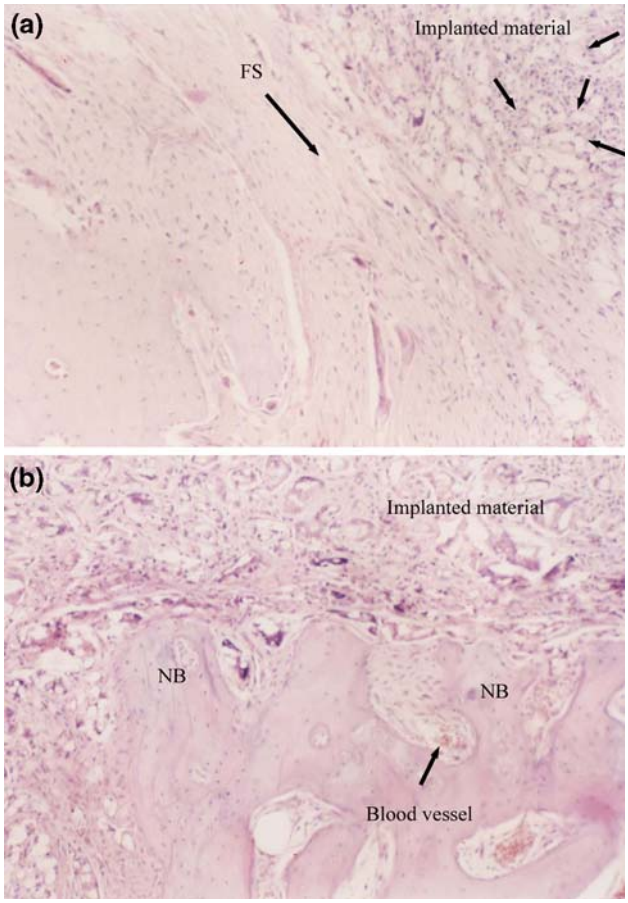

Fig. 6 Histological appearances of rabbit radialis defect implanted with PDLLA and chitosan-modified PDLLA scaffolds after 8 week (H&E 100×): (**a**) PDLLA scaffold, sever inflammatory reaction, lymphocytes, plasma cells, macrophages and foreign-body giant cells were observed (arrow). An obvious connective tissue fibrous structure (FS) surrounded the material space was noticed; and (**b**) chitosan-modified PDLLA scaffold. New bone (NB) formed inside implanted materials that had been surface-modified with chitosan. Only a moderate inflammatory reaction was observed

implantation stage, new blood vessels were observed inside the chitosan surface-modified scaffolds.

Eight weeks

Figure 6a shows the histological observations of the native PDLLA scaffolds after implantation for 8 weeks. No bone and osteoid matrix formation were found inside the implanted scaffolds. Part of the implanted scaffolds collapsed due to degradation. Severe inflammatory reaction took place in the scaffolds with lymphocytes, plasma cells, macrophages and foreign-body giant cells clearly observed. We assume that this severe inflammatory reaction is related to the fact that the PDLLA polymer degraded seriously at this stage, resulting in the high local concentration of acidic degraded products. Around the scaffolds, an obvious connective tissue capsule was observed. Compared to the control group, new bone was observed inside the chitosan surface modified scaffolds (Fig. 6b). This observation reflects good tissue compatibility between the chitosan surface modified scaffolds and the surrounding tissues. For the surface-modification group, only a moderate inflammatory reaction was observed despite of the clear degradation of the scaffolds.

Twelve weeks

In the control group, the connective tissue capsule surrounding the scaffolds developed at eight weeks still existed. Only some small rod-like new bone formed between the original bone and the connective tissue capsule (Fig. 7a). The inflammatory reaction became lighter as

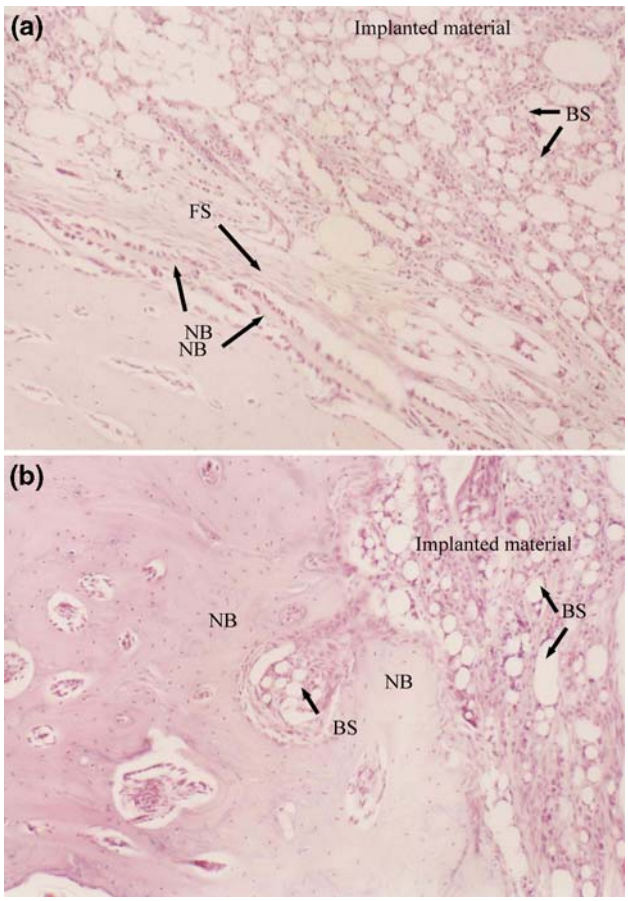

Fig. 7 Histological observations of rabbit radialis defect implanted with PDLLA and modified PDLLA scaffolds after 12 week (H&E 100×): (**a**) PDLLA scaffold. Some rod-like (arrow) new bone formed between original bone and connective tissue capsule (TC). Many bubble-like structures (BS) were present inside implanted materials Light inflammatory reaction was observed; and (**b**) chitosan-modified PDLLA scaffolds. Much new bone (NB) was formed which were surrounded with implanted materials

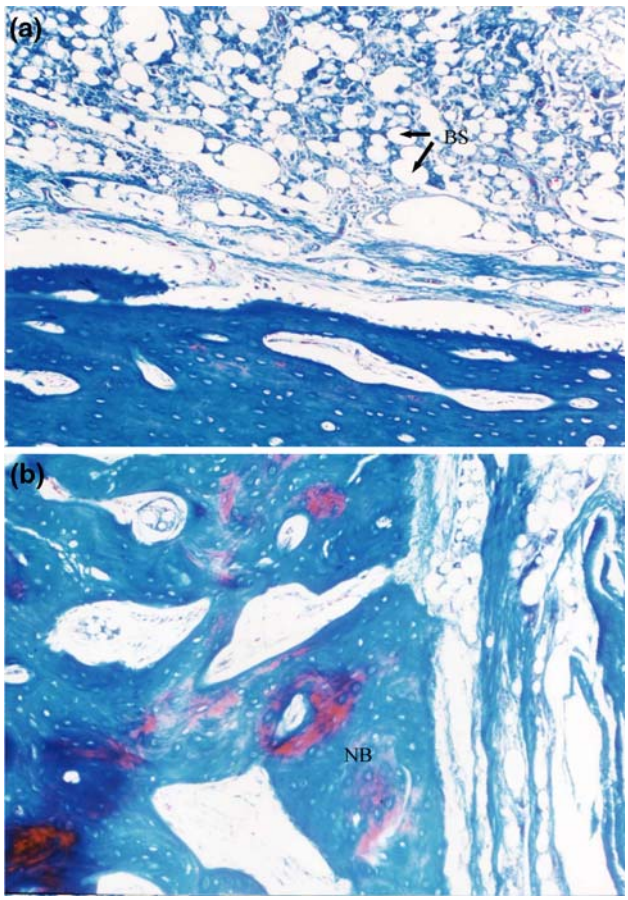

Fig. 8 Histological appearances of rabbit radialis defect implanted with PDLLA and chitosan-modified PDLLA scaffolds after 12 week (Masson 100×): (**a**) PDLLA scaffold, rod-like (arrow) new bone formed between original bone and connective tissue capsule; and (**b**) chitosan-modified PDLLA scaffold. It displayed the remodeling process of new bone (red); some remaining implanted materials (RM) were surrounded by new formed bone

compared with that at 8 weeks. Inside the scaffolds, much space developed at this stage. Fig. 7b shows the chitosan surface modified PDLLA scaffolds after implantation for 12 weeks. A lot of new bone was formed inside the scaffolds at this stage with slight inflammatory reaction. Some new bone even connected together to form circles around the scaffolds, which were still not completely adsorbed. This result indicates good interface between the newly formed bone and the implanted chitosan surface-modified scaffolds. Figure 8 shows the histological observations of both surface-modified and native PDLLA scaffolds that were stained with Masson. In the control group, the sample nearly displays the same appearance as shown in Fig. 7a. However, in the surface-modified group, the sample displays a remodeling process of new bone (red) that surrounds the remaining scaffold.

Discussion

Osteocalcin, bone-carboxyglumatic acid-containing protein (BGP), is one of the components of bone. This protein correlates with the activity of osteoblast during the bone formation process [22]. Therefore, the amount of the osteocalcin in serum could reflect the bone formation potential. The osteocalcin production results in the present work shows that the surface modification of PDLLA scaffolds with chitosan is beneficial to the formation of bone as compared with the unmodified scaffolds.

The inflammatory reactions of both native and surface modified PDLLA scaffolds implanted in radialis defect were light after 2 weeks of implantation. At the implantation time of 8 weeks, the inflammatory reaction became moderate for the surface-modified scaffolds and severe for the native scaffolds, respectively. These experimental

results could be ascribed to the degraded acidic products of the scaffolds, which accumulated to a great amount at the time of eight weeks to induce obvious tissue reaction. Similar result has also been reported by Ekholm et al. [4]. Further, the histological observations have shown that the bone formation potential in the surface-modified PDLLA scaffolds were greatly higher than that in the native scaffolds. As a conclusion, the surface modification of PDLLA scaffolds using chitosan has obvious positive effects for the induction of new bone formation. The possible mechanism of chitosan in the accelerating of new bone formation in the PDLLA scaffolds could be contributed to the physiological property of chitosan. Chtiosan is derived from chitin, which is the major structural molecule in arthropod cuticles. Chitosan is a binary polyheterosaccharide of N-acetyglucosamine and glucosamine in a $\beta 1 \rightarrow 4$ linkage. In structure, it resembles glycosaminoglycans, which are major components of extracellular matrix of bone and cartilage. Glycosaminoglycans links to a protein core and are organized to form proteoglycans. In that environment, the interactions between the matrix proteins and the connective tissue components are numerous and undoubtedly intimate. Therefore, the resemblance of chitosan to components of proteoglycans might be conducive to cell attachment and in turn stimulate cell functions. Previous researches have confirmed that such complex molecules played a key role in modulating cell morphology, proliferation and differentiation [23, 24]. In another study, Frondoza et al. [25] confirmed that chitosan supported the expression of extracellular matrix proteins in human osteoblasts and chondrocytes. The experimental results of this work further confirmed that surface modification of scaffolds with chitosan is helpful to improve the tissue compatibility of the PDLLA scaffolds and therefore contributes to the bone formation.

Conclusion

In the present study, chitosan was employed to surface modify 3-D PDLLA scaffold; surface morphology and histological study was performed to assess its behavior in vivo. Surface morphology and mechanical property of PDLLA scaffold were not changed during surface modification process. Histological results displayed that surface modification of PDLLA with chitosan was beneficial to its bone formation. In combination of our previous study [14], this work further revealed that chitosan, as a helpful agent for surface modification of scaffolds, could be widely used in bone tissue engineering.

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