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Modification of bone graft by blending with lecithin to improve hydrophilicity and biocompatibility

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Abstract

Lecithin was blended to improve the hydrophilicity and biocompatibility of bone graft containing poly(L-lactic acid) (PLLA). Solution blending and freeze drying were used to fabricate symmetrical scaffolds containing different percentages of lecithin (lecithin: PLLA = 0, 5, 10 wt%). Scanning electron microscopy showed that the scaffolds maintained the three-dimensional porous structure. A water uptake experiment proved the significant improvement of hydrophilicity of the blend scaffold. With the addition of lecithin, the compressive strength and compressive modulus decreased. When the weight ratio of lecithin to PLLA was up to 10%, the compressive strength was still more than the lower limit of natural cancellous bone. To test the biocompatibility of the scaffolds, cell culture *in vitro* and subcutaneous implantation *in vivo* were performed. MC3T3-E1 preosteoblastic cells were cultured on the scaffolds for 7 days. Methylthiazol tetrazolium assay and laser scanning confocal microscopy were used to exhibit proliferation and morphology of the cells. The subcutaneous implantation in rats tested inflammatory response to the scaffolds. The results proved the better biocompatibility and milder inflammatory reactions of the blend scaffold (lecithin: PLLA = 5%) compared with the scaffold without lecithin. The modified scaffold containing lecithin is promising for bone tissue engineering.

(Some figures in this article are in colour only in the electronic version)

Introduction

Large bone fracture defects or bone tumor resections are serious problems for bone surgery. Bone grafts are often required to help repair or replace damaged or diseased bone [1]. Ideal bone grafts should have good biocompatibility, biodegradability, porous structure and enough mechanical stability. The chemical composition and physical properties of the biomaterials used in the fabrication of a scaffold are the key factors in determining its performance properties [2]. Many researchers have constructed various scaffolds for bone tissue engineering [3–12]. In our previous work, we developed biodegradable bone scaffold materials containing collagen or recombinant human-like collagen, hydroxyapatite and poly(L-lactic acid) (PLLA) by biomimetic synthesis [13–16]. It was proved that the porous scaffolds were efficient materials for bone tissue engineering. When combined with rhBMP-2 or other growth factors, the scaffolds had promise for the clinical repair of large bony defects. However, one significant limitation of these scaffolds is the lack of hydrophilicity since the materials are hydrophobic, especially PLLA. Water-binding ability of the scaffolds is an important feature to evaluate biomaterial properties for tissue engineering and a hydrophobic material has been known to be unfavorable for cell attachment [17, 18].

Lecithin is a natural mixture of phospholipids and neutral lipids. Phospholipids possess a charged head group and a hydrocarbon tail that contain various amounts of

unsaturation. Because of their amphiphilic chemical structure, phospholipids organize into a bilayer matrix and serve as the building block of cell membranes [19, 20]. Zhu *et al* have blended lecithin with PLLA to enhance the biocompatibility, hydrophilicity and toughness while maintaining mechanical strength of PLLA [21]. It is a promising method to modify scaffolds containing polymers which lack suitable surface properties.

In the present work, solution blending and freeze drying were used to fabricate symmetrical bone scaffolds containing different percentages of lecithin. The hydrophilicity of the scaffolds was improved significantly while the porous three-dimensional structure was maintained. The mechanical properties of the blend scaffold were tested. Cell culture and subcutaneous implantation proved the improvement of biocompatibility of the modified scaffold.

Materials and methods

Materials

Lecithin of medical purity from egg yolk was used. Water-soluble recombinant human-like collagen (RHLC) was developed by recombinant DNA and had the characteristic of Gly-X-Y triplet. It was produced by cloning a partial cDNA that was reversed by mRNA from human collagen $\alpha 1$ (I) and transferred to *E. coli* (Xi'an Giant Biogene Technology (XABTC) Co. patent number: PCT/CN02/00424). Zhai reported the amino acid sequence of this recombinant human-like collagen [22]. Poly(L-lactic acid) with an average molecular weight of 100 000 Da was purchased from the ShangDong Medical Research Institute of China. $CaCl_2$, H_3PO_4 and NaOH were of analysis grade. As a solvent, deionized water was used.

Fabrication

The scaffolds were fabricated using a process very similar to that described by Liao *et al* [15]. The RHLC was diluted in deionized water at a concentration of 2 mg ml^{-1} at room temperature for 3 h. Solutions of $CaCl_2$ and H_3PO_4 (Ca/P = 1.67) were then added separately by drops. The solution was gently stirred and the pH was adjusted to 7.4 at room temperature with sodium hydroxide solution. After 24 h, the nHAp/RHLC deposition was harvested by centrifugation and freeze dried. PLLA was completely dissolved in 1,4-dioxane and stirred for 24 h at room temperature to make an 8% (w/v) solution. Then lecithin was added into the PLLA solution. The weight ratios of lecithin to PLLA were 0%, 5% and 10% (named 0%L scaffold, 5%L scaffold and 10%L scaffold). The lecithin was blended by gently stirrng to form a uniform solution. Then the nHAp/RHLC powder was added at a 1:1 nHAp/RHLC:PLLA weight ratio. After homogenization, the mixture was poured into column-shaped molds, frozen at a temperature −20 °C overnight and then lyophilized to remove dioxane.

Characterization

Morphology. Scaffold morphology and microstructure were examined by scanning electron microscopy (SEM). Samples were sputter coated with a layer of gold about 10 nm thick for SEM (JSM-6301F) observations at 20 kV.

Water uptake experiments. Dried scaffolds (4 mm × 3 mm × 1.5 mm, obtained using a vacuum oven at 37 °C overnight) were accurately weighed (W_0) and incubated in distilled water at 37 °C. At the scheduled time, the masses of the samples were recorded after the surface water was adsorbed by a filter paper (W_t). The water uptake ratio was calculated as follows:

$$\text{Water uptake ratio} = [(W_t - W_0)/W_0] \times 100$$

Each value was averaged from ten parallel measurements [23, 24].

Mechanical properties. The compressive mechanical property was tested with a ZWICK Z005 mechanical tester with a 5 kN load cell. The specimens were cylinders about 9 mm in diameter and about 13.5 mm in length. The cross-head speed was set at 1 mm min^{-1}, and the load was applied until the cylinder was compressed to more than 30% of its original length. The compressive modulus was calculated as the slope of the initial linear portion of the stress–strain curve. The compressive strength was determined as the 30% strain point.

Cell culture

MC3T3-E1 (a clonal osteogenic cell line derived from newborn mouse calvarias, which is often used in bone tissue engineering research) [25] cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin at 37 °C in a 5% CO_2 incubator [15, 21]. The composites were cut into disks (thickness 1 mm) sized for 48-well tissue culture polystyrene plate (TCPS) wells. The cells were seeded into the sterilized composite at a concentration of 5×10^4 cells/well. The cultures were maintained in a humidified atmosphere consisting of 95% air/5% CO_2 (v/v) at 37 °C, and the medium was changed every 2 days.

Proliferation. After 1, 3, 5 and 7 days, a methylthiazol tetrazolium (MTT) assay was used to quantitatively assess the cell metabolic activity ($n = 3$). The culture medium was then replaced with serum-free culture medium containing MTT (0.5 mg ml^{-1}). Following 4 h incubation at 37 °C in an air atmosphere containing 5% CO_2, the supernatant was replaced by 200 μl of DMSO. The plates were shaken for 10 min to ensure the complete dissolution of the Formazan. 100 μl solution of each sample was placed in a microtiter plate and the optical densities at 490 nm were measured on a SS-3000 immunoanalyzer.

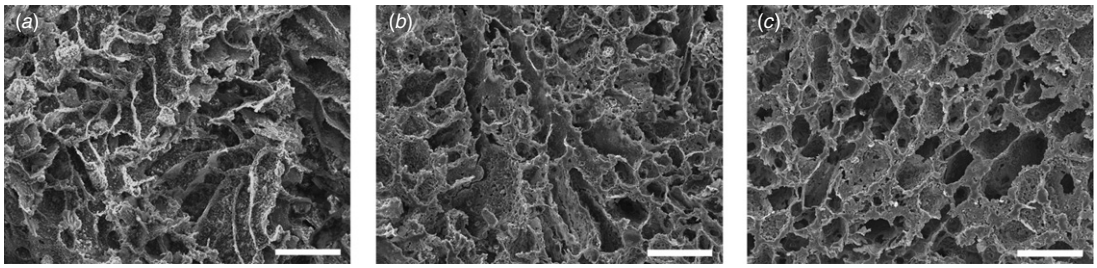

Figure 1. SEM morphology of interconnected porous structure of scaffolds. (*a*) 0%L scaffold, (*b*) 5%L scaffold and (*c*) 10%L scaffold. Scale bar is 200 μm.

Morphology observation. The cells adhering to the scaffolds were washed with phosphate buffered saline (PBS) after 7 days of culture and then fixed with 4% formaldehyde in the PBS for 0.5 h. After being thoroughly washed with PBS, the cells were dyed with propidium iodide (PI) for 30 min. Images were taken with laser scanning confocal microscopy (LSCM). The technique was used to observe the morphology and adhesion of the cells in the scaffolds.

Implantation

Six-week-old male Kunming mice were housed in sterilized cages with sterile food and water and filtered air. NIH guidelines for the care and use of laboratory animals were observed. Each mouse was subcutaneously implanted (dorsal region) with three kinds of scaffold disks (0%L, 5%L, 10%L; thickness 1 mm; ∅ 5 mm). The back of each aether-anesthetized mouse was shaved and disinfected with chlorhexidine, three incisions were made and subcutaneous pockets were created. Then the scaffolds were implanted. At day 2 and day 12 after implantation, the mice were sacrificed, the implanted areas were dissected and the implant-containing tissues were removed from the subcutaneous dorsum. The tissue samples were fixed in 10% formalin and then embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for a histological analysis.

Statistical methods

An unpaired *t*-test was used to compare the results. Repeated-measure STATA was employed to analyze the data for each of the character parameters. In all evaluations, $p < 0.05$ was considered as statistically significant.

Results and discussion

Morphology of the three-dimensional scaffolds

Figure 1 shows SEM images of freeze dried scaffolds containing different percentages of lecithin (lecithin: PLLA = 0, 5, 10 wt%). The three kinds of scaffolds had the same morphology and exhibited the desired three-dimensional interconnected porous structure. The nHAp/RHLC grains are uniformly distributed throughout the scaffolds. The result showed that PLLA and lecithin were miscible and no phase separation appeared during the fabrication process. Previous research proved the miscibility of PLLA and lecithin [21]. Our result was consistent with that and demonstrated that the scaffolds containing lecithin were fabricated successfully using the solution blending and freeze drying method.

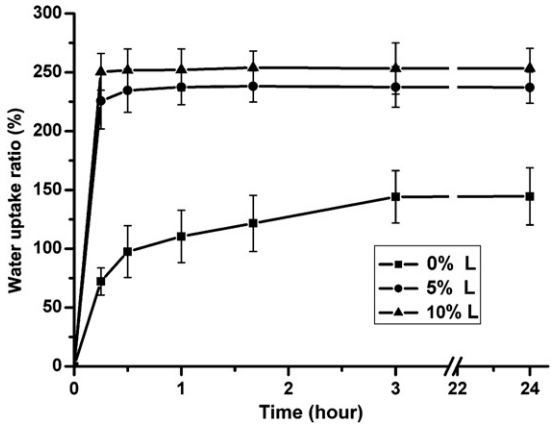

Figure 2. Water uptake ratio–time plots of 0%L scaffolds, 5%L scaffolds and 10%L scaffolds.

Hydrophilicity

The hydrophilicity of tissue engineering scaffolds is very important for their application. Many works have reported that cells attached and spread more easily and effectively on surfaces with proper hydrophilicity than on hydrophobic surfaces [26–28]. The hydrophilicity of the control scaffold (0%L) and blend scaffolds (5%L, 10%L) were studied using a water uptake experiment. The water uptake ratios of the three kinds of scaffolds at different immersion times are shown in figure 2. It was seen that the blend scaffolds attained a maximum water uptake ratio within initial 30 min and then stopped taking up water. The control scaffold took up water slowly and attained its maximum after about 3 h. The water uptake ratios of saturated scaffolds were 144 (0%L), 237 (5%L) and 253 (10%L) separately. The water absorption power of the blend scaffolds was obviously enhanced as compared with the control scaffold.

Lecithin is a natural mixture of phospholipids and neutral lipids. Phospholipids possess a positively charged head group and a hydrocarbon tail that contains various amounts of unsaturation [19, 20]. The amphiphilic chemical structure

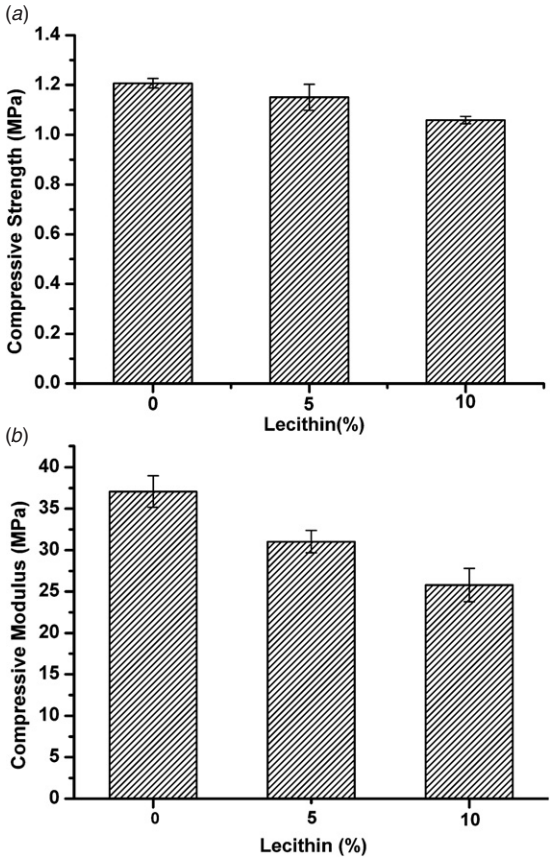

Figure 3. The compressive property testing results of the scaffolds. (*a*) The compressive strength value of 0%L, 5%L and 10%L scaffolds and (*b*) the compressive modulus of 0%L, 5%L and 10%L scaffolds.

of lecithin led to the miscibility of PLLA and lecithin and the hydrophilic nature of the blend scaffold.

Mechanical properties

Mechanical properties are important in determining the potential performance of new materials expected to be used in biomedical applications. In this study, the compressive mechanical properties of control and blend scaffolds were tested (figure 3). The compressive strength and compressive modulus decreased with the addition of lecithin. In the blend scaffolds, the addition of flexible lecithin chains enhanced the segmental mobility of PLLA chains [21] and caused the decrease of compressive strength and compressive modulus. The compressive strength of 10%L scaffolds was still more than the lower limit of natural cancellous bone (1 MPa) [29].

Cell culture

To evaluate cell proliferation, the cell viability within different scaffolds was compared. MTT assay values of the MC3T3 cells on/in the three kinds of scaffolds on days 1, 3, 5 and 7 are shown in figure 4. The proliferation of the cells cultured on the 5%L scaffolds was significantly enhanced compared with 0%L scaffolds. The difference between the two kinds of

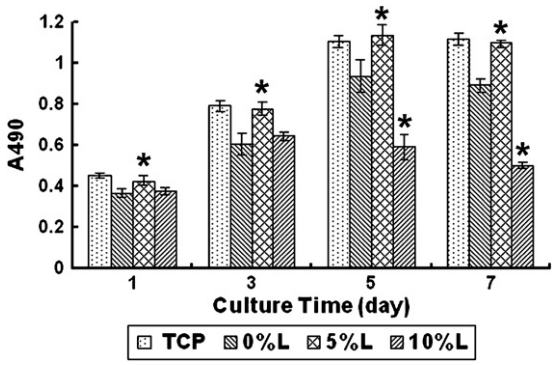

Figure 4. MTT assay after MC3T3 cells were cultured in scaffolds for 1, 3, 5 and 7 days. Asterisks indicate significant differences between blend scaffolds and 0%L scaffolds ($p < 0.05$).

scaffolds was significant during the whole period of culture ($p < 0.05$). The MTT adsorption of the 5%L scaffold was almost equal to that on the TCPS. And the highest value was observed at 5 day incubation. At 1 day and 3 day incubation, the adsorption values of the 10%L scaffold and 0%L scaffold were not significantly different. At day 5 and day 7, the values of the 10%L scaffold decreased and were much smaller than those of the 0%L scaffold.

LSCM was used to observe the morphology and proliferation of MC3T3 cells on/within the scaffolds *in vitro*. Figure 5 shows the LSCM images of the cells cultured for 7 days. The cells in the three kinds of scaffolds had two typical morphologies: spindle shape and polygonal shape, indicating that the cells had entered into a complete adherence stage [30]. The cells on the 10%L scaffold spread better than those on the 0%L scaffold and 5%L scaffold. The qualitative number of cells on the 5%L scaffold was more than that on the 0%L scaffold. The 10%L scaffold showed a significantly smaller number of cells. The result was accordant with that of MTT.

The positively charged groups of lecithin could enhance the interaction between the PLLA surface and the negatively charged cells. But the results of cells on the 10%L scaffold were negative. These results were consistent with the previous report [21]. There are two possible reasons to explain the weak biocompatibility of the 10%L scaffold. The first possible reason is that different types of cells adhered, spread and grew more onto the positions with moderate hydrophilicity of the wettable surface than onto the more hydrophobic or hydrophilic positions [28]. The oxidation of lecithin is another possible reason. The oxidation of phospholipid polyunsaturated acyl chains is thought to result in some way in an impairment of the associated protein activity [31].

In vivo analysis

The implantation of a biomaterial leads to a response to injury that activates mechanisms of healing the damaged tissues [32]. Since the effective use of biomaterials *in vivo* requires good biocompatibility and biofunctionality, it is vital to assess and compare the inflammatory reactions provoked by the implanted scaffolds. The subcutaneous implantation in mice

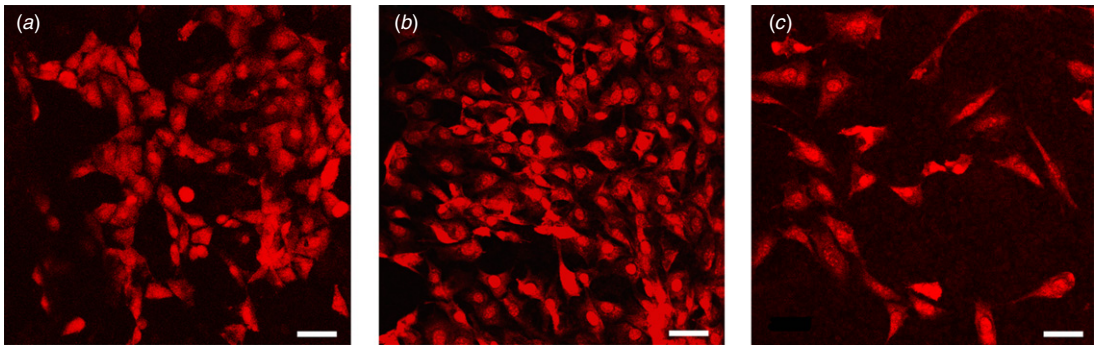

Figure 5. LSCM observation of MC3T3 cells cultured for 7 days within 0%L scaffolds (*a*), 5%L scaffold (*b*) and 10%L scaffold (*c*). Scale bar is 20 μm.

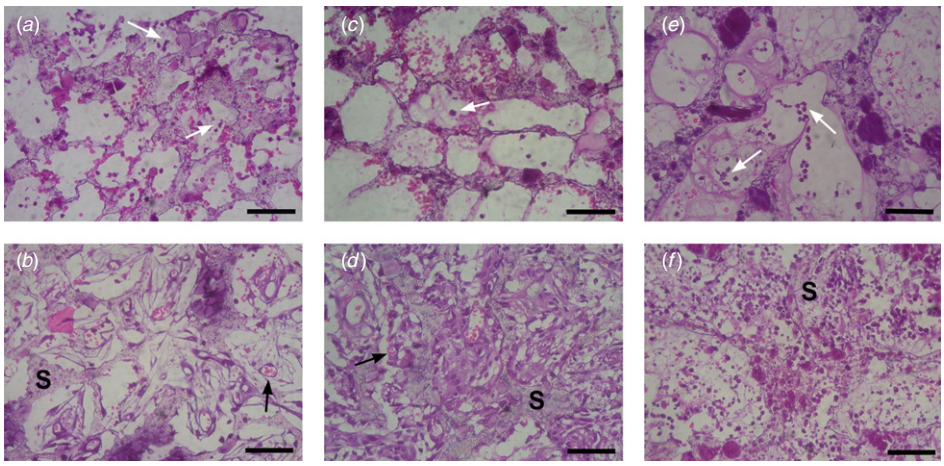

Figure 6. Histology H&E staining of subcutaneous scaffolds and tissue: (*a*) 0%L scaffold, 2 day; (*b*) 0%L scaffold, 12 day; (*c*) 5%L scaffold, 2 day; (*d*) 5%L scaffold, 12 day; (*e*) 10%L scaffold, 2 day and (*f*) 10%L scaffold, 12 day. White arrows point to inflammatory cells. Black arrows mark the newly formed capillary vessel. S: scaffold. Scale bar is 100 μm.

was performed. The representative histological sections of tissue samples are shown in figure 6.

At 2 days post-implantation, all the scaffolds showed infiltration of inflammatory cells (figures 6(*a*), (*c*), (*e*)). The 5%L scaffold resulted in the least accumulation of inflammatory cells and did not provoke a significant inflammatory response. At 12 days after implantation, the prominent infiltration and ingrowth of host cells occurred in the 5%L and 0%L scaffolds. There were many activated fibroblasts that grew into the scaffolds but almost no inflammatory cells. Meanwhile, prominent neovascularization could be seen (figures 6(*b*) and (*d*)). The proliferation of fibroblasts, synthesis of collagen and angiogenesis were characteristic of the healing response of inflammation [32]. When the sections of these two scaffolds were compared, it could be seen that fibroblasts and collagen in the 5%L scaffold were more abundant than those in the 0%L scaffold, indicating the better biocompatibility of the 5%L scaffold. In the section of the 10%L scaffold, there were numerous inflammatory cells but almost no fibroblasts, showing the chronic inflammation but no healing response (figure 6(*f*)). The better biocompatibility of the 5%L scaffold was proved by combining the results of experiments *in vitro* and *in vivo*.

Conclusion

The new bone graft scaffold containing lecithin was fabricated successfully using the solution blending and freeze drying method. Lecithin was homogeneously mixed with PLLA and significantly improved the hydrophilicity of the scaffold. The compressive strength and compressive modulus decreased with the addition of lecithin. The compressive strength of 10%L scaffolds was still more than the lower limit of natural cancellous bone. In the MC3T3 cell culture tests, the modification of cellular proliferation and attachment on the scaffold depended on the percentage of lecithin, and the ideal weight ratio of lecithin to PLLA is 5%. The subcutaneous implantation exhibited the minimum inflammatory reaction and the best biocompatibility of the 5%L scaffold compared with the 10%L scaffold and control scaffold without lecithin. The blend scaffold with lecithin is a promising biomaterial for bone tissue engineering.

Acknowledgments

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