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Fabrication and characterization of nano composite scaffold of poly(L-lactic acid)/hydroxyapatite

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Abstract To mimic the nano-fibrous structure of the natural extracellular matrix, a nano composite scaffold of poly(L-lactic acid)/hydroxyapatite(PLLA/HAP) was fabricated by a thermally induced phase separation method. The characterization of the composite scaffold showed that the scaffold had a nano-fibrous PLLA network (fiber size 100–750 nm), an interconnective microporous structure (1–10 μm) and high porosity (>90%). HAP was homogeneously distributed in the scaffold, as a result, the compressive modulus of PLLA/HAP (80:20, w/w) increased to 3.15-fold compared with that of a pure PLLA scaffold. Incorporating HAP into PLLA network also buffered the pH decline in vitro degradation and enhanced the protein adsorption of the composite scaffold significantly. The new nano composite scaffold is potentially a very promising scaffold for tissue engineering.

1 Introduction

The goal of tissue engineering is to repair, or replace, the function of defective or damaged tissues or organs. One of the key factors is the creation of scaffold as an artificial extracellular matrix (ECM) for cellular attachment, proliferation and differentiation [1]. To perform these functions in tissue engineering, the scaffolds should meet certain requirements of biocompatibility, biodegradability, optimal mechanical property, adequate porosity and morphology for tissue cultivation. Collagen is one of the most important components in natural ECM, and is present in bone, skin, tendon, ligament, and other tissues. Collagen fiber bundles vary in size from 50 to 500 nm [2, 3]. A nano-fibrous scaffold used as an artificial ECM with interconnective pores and large surface areas enhances cell adhesion, migration, proliferation, and differentiation. This should therefore serve as a better environment for cell function [4, 5]. To mimic the architecture of a natural ECM, many researchers have produced nanoscale scaffolds and validated that cells attach and organize well around these scaffolds [6, 7]. However, nanoscale scaffolds are usually weaker than the microscale scaffolds and this limits their applications in tissue engineering [8].

Methods to produce nano-fibrous scaffolds include self-assembly, electrospinning, and phase separation. Self-assembly production can generate small diameter nano-fibers in the lowest end of the range of natural extracellular matrix collagen [9]. Electrospinning only produces randomly oriented and interconnected voids, often resulting with the electrospun fibers being in the micrometer range instead of a nanometer one [10, 11]. Phase separation generates nano-fibers in the same range as natural extracellular matrix collagen and allows for the designed pore size and porosity [11]. The phase separation method is therefore becoming more important in the fabrication of nano-fibrous scaffolds.

Many of the porous scaffolds studied to date were composed of biodegradable polymers and bioactive ceramics. Poly-L-lactic acid (PLLA)/hydroxyapatite (HAP) composite became an important representative of these materials since they combined the ostesconductivity and bone bonding ability of HAP with the resorbability and the processing ease of the PLLA [12–16]. It was reported that incorporating HAP into a polymer network improved the activity and viability of cells culture on them, buffered the acidic degradation products from the polyester, and

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improved certain mechanical properties [14–16]. Ma et al. also reported that HAP could impart osteoconductivity to the highly porous PLLA/HAP composite scaffolds for bone tissue engineering and improve the protein adsorption capacity [17, 18]. Previous research suggests that the composite of PLLA and HAP could be an ideal scaffold in tissue engineering. However, these PLLA/HAP scaffolds were not nano-fibrous structures. In order to mimic a natural ECM, this study used a nano-fibrous composite scaffold of PLLA/HAP, fabricated using a thermally induced phase separation (TIPS). The morphology, porosity, compressive modulus, pH changes in vitro degradation and protein adsorption of the scaffold were also investigated.

2 Materials and methods

2.1 Materials

Nano HAP particles were prepared in our lab by a chemical precipitation and hydrothermal technique [19]. Briefly, 0.09 M aqueous solution of $(NH_4)_2HPO_4$ was added drop wise to 0.15 M aqueous solution of $Ca(NO_3)_2$ under a stirring condition to form a reaction solution. The pH of the solution was adjusted to ten by the addition of NH_4OH, and the temperature was maintained at 90 ± 5°C. The precipitate slurry was stirred for 24 h and subsequently aged for 8 h in reaction vessel at 80–90°C. The resulting product was centrifuged and vacuum dried. The XRD pattern of the nano HAP showed peaks at $2\theta = 25.70°$, 28.54°, 31.62°, and 49.32°, matched with the JCPDS card of HAP. TEM analysis of the nano HAP revealed rod-like particles with length between 30 and 180 nm and width of approximately 20–30 nm.

PLLA with an inherent viscosity of 1.6 dl/g (MW = 56000) was purchased from Shandong Medical Appliance Company, China. Other reagents, such as tetrahydrofuran and ethanol, etc., were analytical and purchased locally.

2.2 Fabrication of scaffold

The nano-fibrous PLLA scaffold was fabricated by TIPS method [20]. Briefly, PLLA was first dissolved in tetrahydrofuran in a sealed flask and stirred at 50°C for 20 min to make a homogeneous solution with a concentration of 0.05 g/ml. HAP powder was added into the solution gradually while stirred by magnetic force, and the liquid was dispersed ultrasonically for 20 min. After that, the solution was divided into vials, rapidly transferred into a freezer at −18°C to gel, and kept at the gelling temperature for at least 2 h after gelation. Then, the vials containing the gel were immersed into deionized water for solvent exchange in a refrigerator at −4°C. The water was changed three times a day for 2 days. And then, the gel was removed from the water, and transferred into a freezer at −18°C for at least 2 h. At last, the frozen gel was transferred into a freeze-dryer (FD-1-50,China) under vacuum lower than 30 Pa for 1 week. The dried sample was stored in a desiccator until characterization.

2.3 Characterization of scaffold

The morphology of the scaffold was observed by Scanning Electron Microscope (SEM) (JSM-6390LV, JEOL, Japan) with gold coating by Auto Fine Coater (JFC-1600, JEOL, Japan).

The porosity was measured by a liquid displacement method, in which the liquid of ethanol was used as a wetting agent. The porosity was obtained by $\varepsilon = (V_1 - V_3)/(V_2 - V_1)$, where ε is the porosity of the scaffold, V_1 is the volume of the liquid before the scaffold was put in, V_2 is the volume of the liquid after the scaffold was put in, and V_3 is the volume of the liquid after the liquid was pressed into the pore of the sample and the sample was taken out of the liquid.

The compressive modulus of the scaffold was tested on a universal electronic material testing machine (DXLL-1000, China). A crosshead speed of 0.5 mm/min was used and the size of the dry sample was Φ 20.5 × 5.0 mm. Five specimens were tested for each sample.

2.4 In vitro degradation

A 0.05 g scaffold was put in a vial containing 20 ml normal saline (initial pH was 6.25). The temperature was controlled at 37 ± 1°C. The pH changes were determined by pH meter (Mettler-Toledo, Fiveeasy) for various time periods up to 7 weeks. For each sample, three replicates were taken to calculate the average pH changes.

2.5 Protein adsorption

Protein adsorption was performed by incubating scaffolds (three replicates) in phosphate buffered saline (PBS, 0.1 mol/l, pH = 7.4) containing 2.0% fetal bovine serum (FBS). Before incubation, the samples were pretreated by ethanol for 30 min and then washed by PBS three times for 3 h under gentle stirring. Each scaffold with 0.035 g was put into a vial with 3 ml FBS/PBS solution. The scaffolds were incubated at 37 ± 1°C for a chosen incubation time. The concentration of the protein in the FBS/PBS solution was determined by the method of Bradford with a commercial protein assay kit (Nanjing Jiancheng Biomed. Ltd., China). The amount of proteins adsorbed was calculated by subtracting the amount of proteins left in the FBS/PBS solution after adsorption from the amount of proteins in

control FBS/PBS solution under the same incubation conditions.

3 Results and discussion

3.1 Scaffold morphology

The morphology of the scaffolds was demonstrated by SEM micrographs. Figure 1a and b demonstrated a pure nano-fibrous PLLA scaffold with interconnective pores and large surface area. Figure 1c and d displayed the microstructure of a nano-fibrous PLLA/HAP composite scaffold at 80:20 (w/w). As HAP was added, the nano morphology of the composite scaffold didn't change. It could be seen from these micrographs that the nano-fibrous porous PLLA matrix was well fabricated, in which the diameters of the nano PLLA fibers were about 100–750 nm, similar to these of natural ECM. Nano HAP particles were homogenously dispersed in the scaffolds. Both the nano PLLA fibers and HAP particles had a large surface area, which benefits good in-growth of cells [15, 18]. The structural uniformity of the scaffolds, satisfactory mechanical properties, degradation of the scaffolds, and growth of cells were also favorable [21]. The pore size ranged 1–10 μm. Such a microporous structure enables an efficient supply of oxygen and nutriment to cells [22]. Figure 1e displayed the microstructure of a PLLA/HAP composite scaffold at 70:30 (w/w). The diameter of PLLA fibers was still in the range of 100–750 nm, but the agglomeration of HAP was much greater, thereby reducing the likelihood of being able to form a uniform structure. In all, the nano PLLA/HAP composite scaffolds were successfully fabricated by TIPS method. Also, the microstructure of this scaffold mimicked the natural ECM and provides a better environment for cell growth.

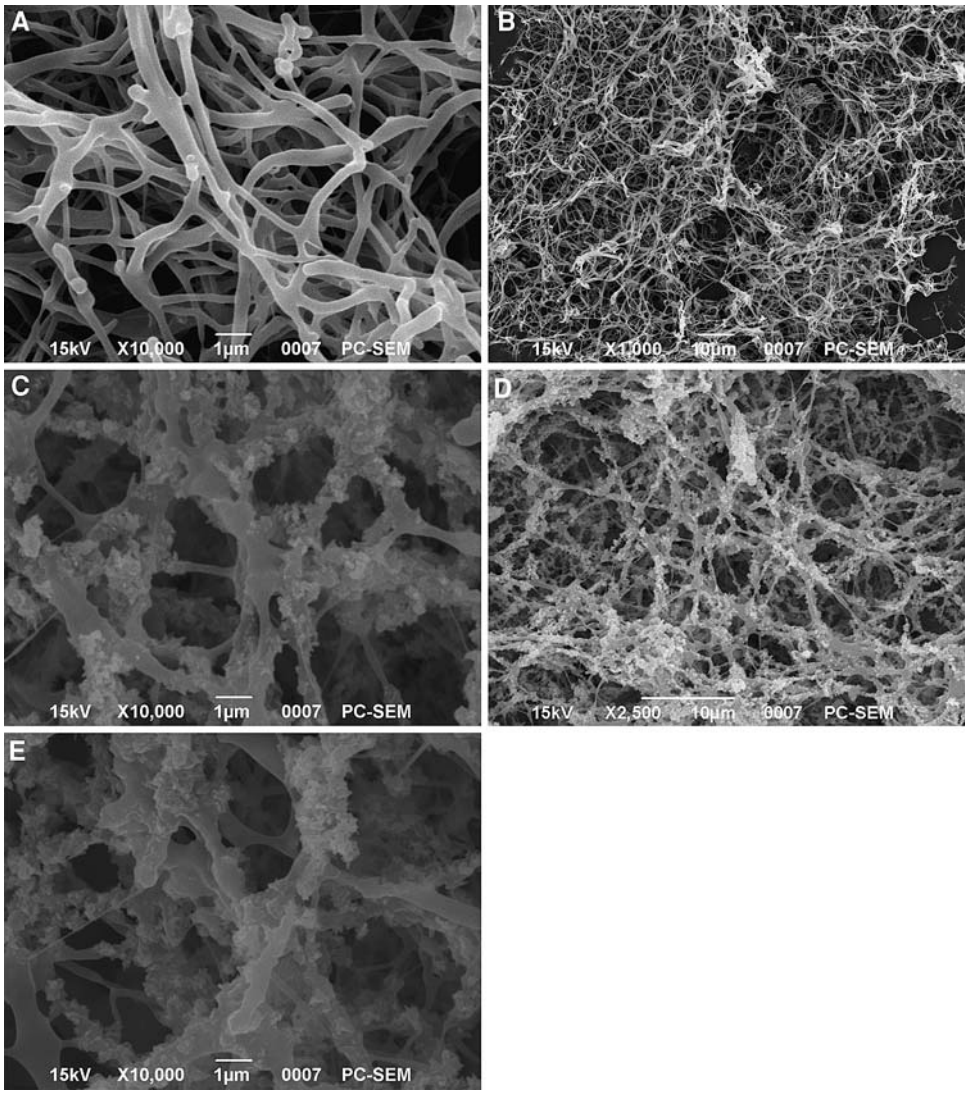

Fig. 1 SEM micrograph of PLLA/HAP composite scaffold. **a** pure PLLA scaffold, ×10,000; **b** pure PLLA scaffold, ×1,000; **c** PLLA/HAP composite scaffold at 80:20 (w/w), ×10,000; **d** PLLA/HAP composite scaffold at 80:20 (w/w), ×2,500; **e** PLLA/HAP composite scaffold at 70:30 (w/w), ×10,000

3.2 Porosity

For scaffolds of tissue engineering, porosity is one of the most important parameters. Porosity of more than 80% is a distinct indicator of an appropriate scaffold [23]. The porosity of the PLLA/HAP composite scaffolds was determined, and the result was shown in Table 1. The slight decrease of porosity, with a high HAP content, was due to the n-HAP particles occupying part of the scaffold pores. From the table, it could be seen that all nano scaffolds in our fabrication had satisfactorily high porosity. Even when the content of HAP was raised to 50%, the porosity of the PLLA/HAP composite scaffold was still greater than ninety percent. Obviously, all these scaffolds had met the demands of porosity for scaffolds.

3.3 Compressive modulus

A good scaffold should not only have appropriate porosity, but also satisfactory mechanical properties, which can keep its shape and characters implanted in the body. The compressive performance of the scaffold is a very important mechanical property because of the necessity of the structural stability to withstand stress incurred during culturing in vitro and implanting in vivo [24]. In our research, HAP with different contents was incorporated into the PLLA network and the compressive modulus of the composite scaffolds was then determined. Figure 2 showed the result of the relationship between the compressive modulus of scaffolds and the contents of HAP embedded in. Generally speaking, introducing HAP into the PLLA network greatly enhanced the compressive modulus of the scaffolds. The compressive modulus of the pure PLLA scaffold was only 0.2 MPa, while the compressive modulus of PLLA/HAP composite scaffold at 80:20 (w/w) was up to 0.63 MPa, which was approximately three times that of the pure PLLA scaffold. However, when more HAP particles were embedded in the scaffold, it caused much greater agglomeration of HAP particles (Fig. 1e). The larger sized HAP particles acted as "flaws" in the continuous PLLA matrix due to limited interfacial bonding with the polymer matrix and limited effects on toughening mechanisms. It could be seen from the figure that the compressive modulus of the composite scaffolds began to decrease when the ratio of HAP to PLLA was more than 20:80 (w/w).

Table 1 Porosity of PLLA/HAP composite scaffolds

PLLA/HAP (w/w)	Porosity (%)
100:0	94.5
90:10	93.7
80:20	93.4
70:30	92.2
50:50	90.5

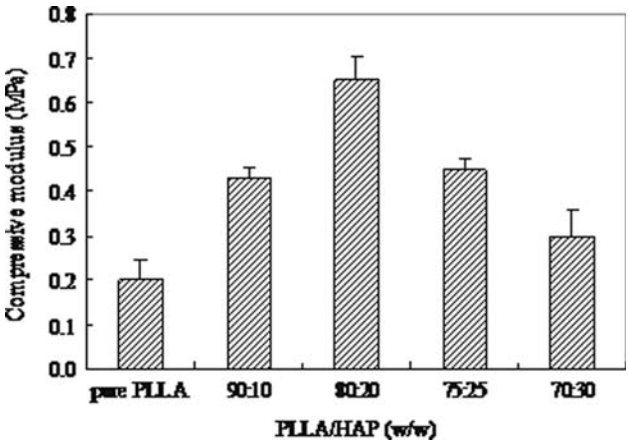

Fig. 2 Effects of HAP contents on the compressive modulus of PLLA/HAP composite scaffolds

3.4 In vitro degradation

All the scaffold samples were incubated in normal saline at 37°C for 7 weeks. The initial pH of normal saline was 6.25. Along with the scaffold degradation in vitro, the pH of the solution was measured. The result is shown in Fig. 3. It can be clearly seen that the pH of the pure PLLA degradation solution decreased remarkably from 6.25 to 5.89 in the first week, while that of the PLLA/HAP composite scaffold increased to 6.66 in the first 2 days, then decreased after that. The increase of pH indicated that the degradation rate of HAP was higher than that of PLLA in the first 2 days. During the testing period, it was obvious that the pH of the PLLA/HAP composite scaffold changed more slowly than that of the pure PLLA scaffold without any HAP. This can be explained as: Firstly, the degradation process of PLLA is known as hydrolysis. Water around the scaffold moves to the surface of the polymer and diffuses into the inner part

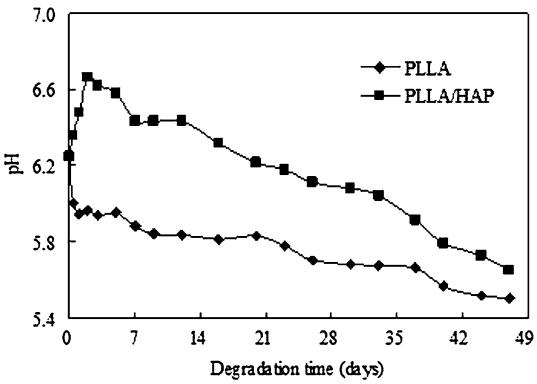

Fig. 3 pH changes of pure PLLA, PLLA/HAP (80:20, w/w) composite scaffold during in vitro degradation

to hydrolysis of ester bond. The hydrolysis increases at a greater rate in the acidic atmosphere. This is caused by the acidic self-catalysis effect [25, 26]. So, the curve of pure PLLA scaffold degradation shows the continuous declining tendency of pH. Secondly, the alkalescent groups of HAP could neutralize the acidic degradation products of PLLA, which made the decline of the degradation solution pH rate less. At the same time, the addition of HAP into the PLLA matrix weakened the acidic self-catalysis effect of PLLA. Therefore, the pH of the PLLA/HAP composite scaffold declined more slowly than that of the pure PLLA scaffold. The improved acidity atmosphere favored the reduction of the indolent inflammatory reaction during the cultivation of the cell and tissue.

3.5 Protein adsorption

The scaffolds of pure PLLA and PLLA/HAP (80:20, w/w) were incubated at 37°C for protein adsorption. The results are shown in Fig. 4. It showed that the protein adsorption of all samples reached equilibrium in 48 h and there was no significant increase of protein adsorption during further incubation. Each 1.0 g pure nano PLLA scaffold could only adsorb 14.6 mg protein. Introducing HAP into the PLLA network significantly increased the protein adsorption with 2.3-fold compared with the pure PLLA scaffolds. The enhancement of protein adsorption could be ascribed to better biocompatibility and bioactivity of HAP (high affinity for proteins), and the nano HAP particles exposed on the surfaces of pore walls provide more total surface area for protein adsorption. High capacity of protein adsorption is an important property of a scaffold in application. The more the protein is adsorbed in a scaffold, the more cells can attach and survive during the initial culture period [27, 28].

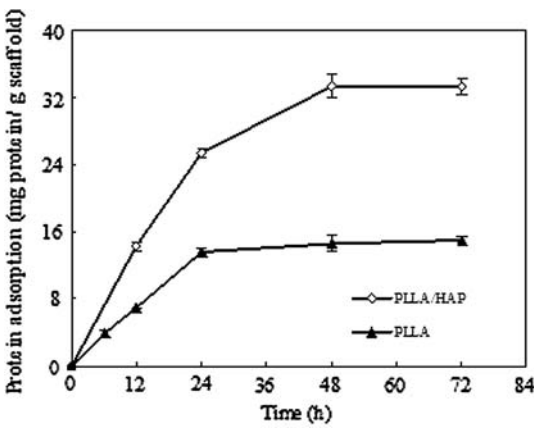

Fig. 4 Protein adsorption curves of pure PLLA scaffold and PLLA/HAP composite scaffold (80:20, w/w)

4 Conclusions

Nano composite scaffolds of PLLA/HAP were successfully fabricated by TIPS method. The morphology showed that the composite scaffolds had nano-fibrous PLLA matrix and homogenously dispersed HAP particles. The composite scaffold had microporous structure and high porosity. Introducing HAP into the PLLA scaffold not only enhanced the compressive modulus of the composite scaffolds at different contents, but also buffered the pH decline in vitro degradation. The resulting scaffolds had better structure stability and less indolent inflammatory reaction during the cultivation of the cell and tissue. Protein adsorption results indicated that the composite scaffolds significantly enhanced the protein adsorption, which benefits cell growth. In summary, the new composite scaffolds show a great deal of promise for use in tissue engineering.

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