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3D PLLA/Nano-hydroxyapatite Scaffolds with Hierarchical Porous Structure Fabricated by Low-temperature Deposition Manufacturing

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Abstract: A new Precision Extrusion nozzle based ball screw transmission was developed. 3D hierarchical porous PLLA/nano-Hydroxyapatite(PLLA/nHA) scaffolds were fabricated by low-temperature deposition manufacturing. Scaffolds with macropores of 200-500 µm and micropores about 10 µm were fabricated through a thorough study and control of the processing parameters, in which the processing path and speed of material extrusion determine the macropores and there is a suitable temperature zone for fabricating qualified macropores. Micropore morphology can be controlled by adjusting supercooling of solvent crystallization or adding water into the solvent system. The compressive modulus of the scaffolds in air and phosphate buffer solution was measured, which increased with HA addition. In-vitro cell culture results showed a good biocompatibility of PLLA/HA scaffolds with the pre-osteoblastic MC3T3-E1 cells.

Key words: tissue engineering; rapid prototyping; low-temperature deposition; scaffold; PLLA; nano-hydroxyapatite

1 Introduction

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function by combining cells with extracellular matrix (or scaffolds)[1,2]. The internal architectures of scaffold, including pore size, pore shape, and pore connection pattern, play important roles for cellular ingrowth, proliferation and new tissue formation. The macropore more than 200 µm is good for vessel growth and getting nutrition in and metabolic wastes out during tissue growth, while scaffold topology or micropore is very important for cell adhesion, migration, and growth etc.. Biomaterials used for scaffold fabrication in bone tissue engineering include natural-derived biomaterials and synthetic biomaterials. Composite materials which combine polyester and hydroxyapatite or bioglass are often used for tissue-engineered bone. The traditional scaffold fabricating method is freeze-drying, solvent-casting particulate-leaching, *etc*. Peter X Ma creates highly porous biodegradable PLLA/HA composite scaffolds by using thermally induced phase separation technique. The highest pore size of scaffold is 200 µm by solid-liquid phase separation and 100µm by liquid-liquid phase separation under the condition of low poly(a-hydroxyl acids) concentration for scaffold preparation[3]. V Maquet fabricated PLGA/bioglass scaffold with solid-liquid phase separation and got similar pore morphology[4]. These macropore less than 200 µm can not meet the requirement of vessel growth and micropore structure could not prepared either in this way.

Here we used a new prototyping manufacturing process named low-temperature deposition manufacturing to make 3D PLLA/HA scaffolds with hierarchical porous structure[5]. It overcomed the disadvantages relating to traditional methods such as small pore and low mechanical property of scaffold. A systemic research on the processing technique was conducted to produce hierarchical porous structure. The compressive modulus of the scaffolds were compared and a preliminary biological evaluation was done with

pre-osteoblastic MC3T3-E1 cells.

2 Experimental

2.1 Materials

Poly(L-lactic acid) (PLLA) with an inherent viscosity of approximately 1.6 respectively were purchased from Jinan DaiGang Biomaterials Company (Shandong, China). PLLA was used without further purification. Nano-hydroxyapatite(AR) were purchased from Sinopharm Chemical Reagent Co.(China). Deionized water was obtained with a Milli-Q water filter system from Millipore Corporation (Bedford,MA). Diethylene dioxide (1,4-dioxane), a good solvent for PLLA, was of analytical grade and used without further purification. PLLA was dissolved into dioxane or 1,4-dioxane/water mixture(v/v=88/12) with concentration of 10%(w/v) assisted with a magnetic stirrer while heating at 70 ℃ for 2 h. After the nano-HA powder was gradually added into the solution, the composite slurry was stirred magnetically for another 3h and then ultrasonically for 30 min.

2.2 Low-temperature deposition process

The low-temperature deposition manufacturing is a new computer-aided manufacturing process below 0 ℃ based on the layer-by-layer manufacturing principle of solid freeform fabrication[6].
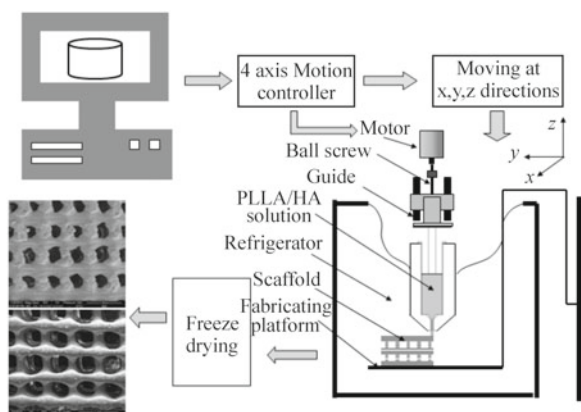

Fig.1 Low-temperature deposition manufacturing for fabrication of 3D PLGA-bioglass scaffolds (a) modeling，slicing, path generation in computer; (b）low-temperature deposition manufacturing in refrigerator

The procedure for fabrication is shown in Fig.1. The manufacturing path was generated in computer which controls nozzle moving in X,Y directions by a 4 axis motion controller. The composite slurry was fed into the nozzle in which an electrical heating rod could keep solution at liquid state, which was extruded out to the refrigerator chamber through a syringe by high-precision ball-screw transmission powered by a step

motor. The computer controls the nozzle of 300 μm diameter moves at X,Y plane while the composite slurry was extruded onto the platform. The liquid slurry was frozen quickly in the refrigerator chamber. After one layer was formed, the platform was moved down in Z direction a layer-thickness of 180 μm. This process was repeated until forming a 3D scaffold at last.

2.3 Freeze-extraction and freeze-drying methods

Two steps, namely the freeze-extraction and freeze-drying, were performed to remove solvent in scaffold. First, the frozen scaffold fabricated in refrigerator was immersed in a water/ethanol(80/20) mixture pre-cooled to −20 ℃ for solvent exchange. The mixture was changed three times a day for 2 days. Then the scaffold was removed from mixture and immersed in de-ionized water for 8 h, and transferred into a freezer at −40 ℃ for at least 2 h. The frozen scaffold was transferred into a freeze-dryer FDU-1100 for 4 days to remove the ice and residual solvents, yielding a highly porous scaffold. The dried porous matrix was then stored in a desiccator until characterization.

2.4 Characterization

The porous morphology of the scaffolds was investigated with scanning electron microscopy (SEM, Quanta, 200F) at 15 kV. Fracture-frozen cross-sections of the scaffold, taken in either longitudinal or transversal way, were coated with gold using a sputter coater. Compressive mechanical properties were studied with an Instron 4502 mechanical tester (Instron Corporation, Canton, MA).

2.5 Cell culture

The MC3T3-E1 osteoblasts from China Center for Type Culture Collection(CCTCC) were cultured in a supplemented α-MEM and 10% fetal bovine serum (FBS) containing 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 37 ℃ with 5% CO_2. The medium was changed every other day. The cells of passages 4 were seeded onto the 3D PLLA/HA scaffolds. After 14 days culture in incubator, the scaffolds with cell were washed with phosphate buffer solution (PBS), fixed with glutaraldehyde for 2 h. A gradual ethanol dehydration was used for water extraction from scaffold and cell.

3 Results and discussion

3.1 Macro-structure of the scaffolds
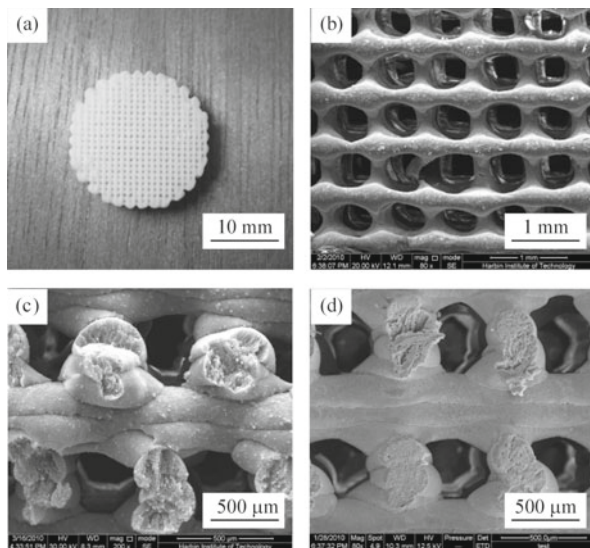
The macropore of scaffold is related to

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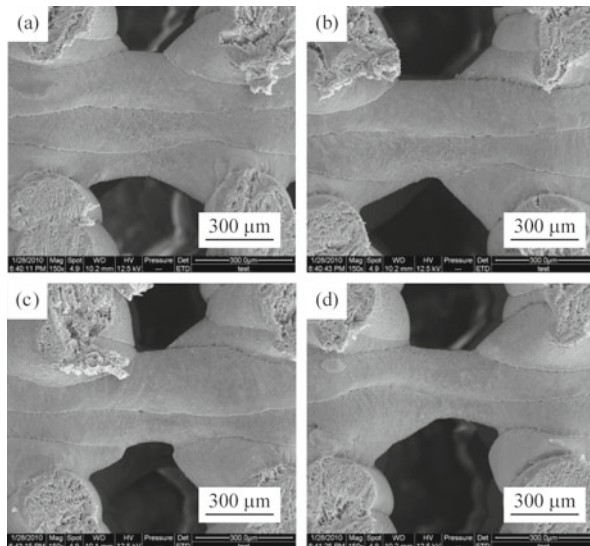
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Fig.2 3D porous PLLA-HA scaffolds

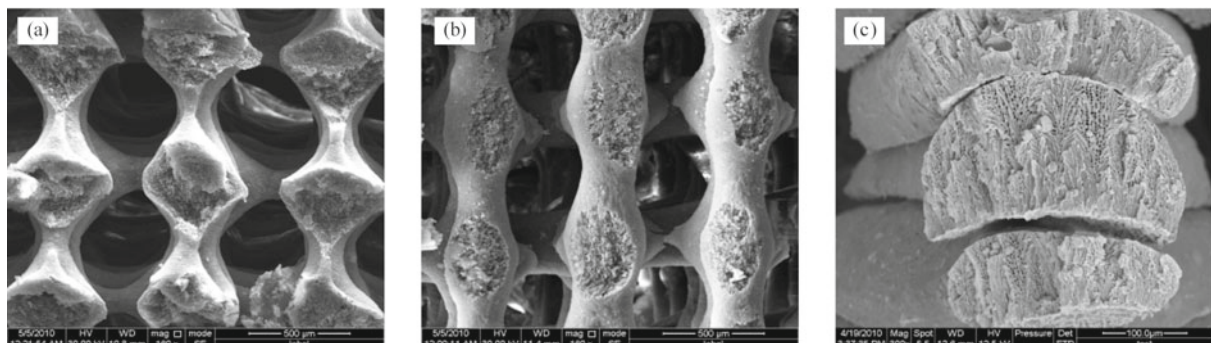Fig.3 SEM images of scaffold fabricated under different nozzle temperatures (a)30℃;(b)35℃;(c)40℃;(d)45℃

permeability which has effect on chondrogenesis by bone marrow stromal cells or vessel growth in bone tissue engineering[7]. Low permeability is need in chondrogenesis and high permeability in vascularization. Up to now only rapid prototyping manufacturing could fabricate scaffold with uniform pores. As shown in the Fig.2, the diameter of strand and space between with two strands was controlled well. In some cell seeding condition, a scaffold with staggered pores was also manufactured such as Fig.2c.

Fig.3 illustrates scaffolds fabricated under different nozzle temperatures when refrigerator temperature was fixed at −10 ℃. A new layer was formed on every three layers at cross direction. The liquid PLLA-HA composite slurry will dump into the line interval of the former three layers if the temperature of refrigerator was not low enough. This may lead to large macropore errors which could even make fabrication fail. So the space between every strand and the temperature of refrigerator chamber are vital parameters of the process.

3.2 Bonding between layers

In terms of strength, the strong bonding between two layers is the key point for a qualified scaffold. Figs.4-6 show different bonding interfaces in different fabricating conditions. All scaffolds were made of 8%(*wt/v*) PLLA slurry with PLLA/HA=5:5(*wt:wt*). The new extruded liquid slurry bonds to the former layer by melting and diffusing into the former layer. So the temperature of nozzle and refrigerator determined the last bonding strength. Fig.4a shows good adhesion and adequate diffusion between the two polymer layers fabricated at 30 ℃ nozzle temperature while maintaining the freezing-chamber at −10 ℃. If the nozzle temperature is too low, the interface of layers may be detached(Fig.4b, 4c). Fig.5 and Fig.6 show the influence of nozzle temperature and freezing-chamber temperature, respectively, on the micro-structures of layers' interfaces. It can be concluded that too low temperature of nozzle or refrigerator was no good for jointing.

Fig.4 SEM images of scaffold fabricated under different nozzle temperatures while refrigerator temperature was fixed at −10 ℃ (a) 30 ℃; (b,c) 15 ℃
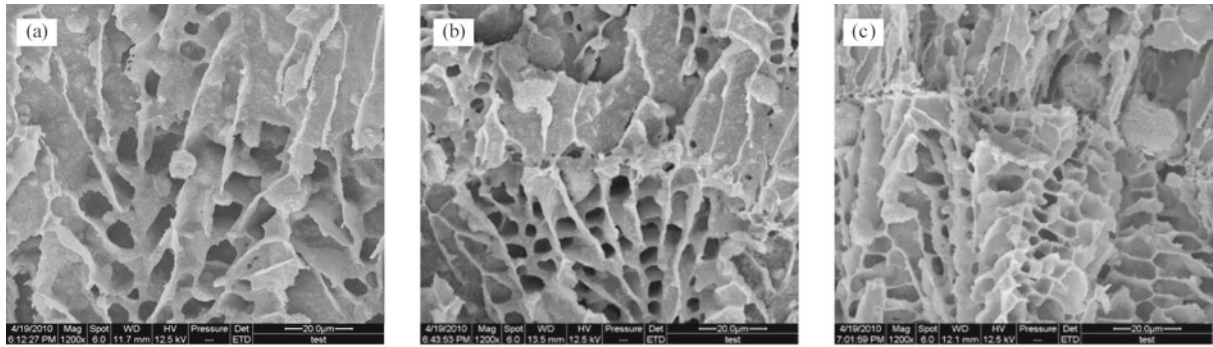
Fig.5　SEM images of scaffold fabricated under different nozzle temperatures while refrigerator temperature was fixed at −10℃ (a)30 ℃; (b) 25 ℃;(c)20 ℃
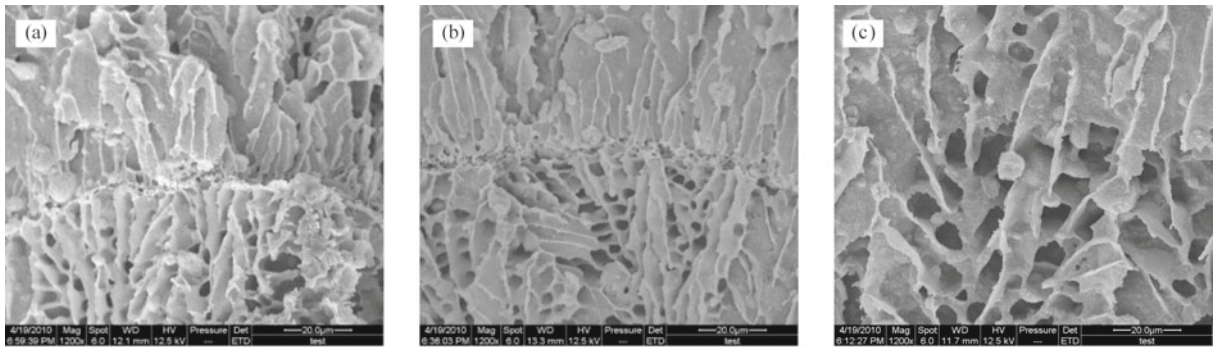

Fig.6　SEM images of scaffold fabricated under different refrigerator temperatures while nozzle temperature was fixed at 30 ℃ (a) −20 ℃; (b) −15 ℃; (c) −10 ℃

3.3　Micropore morphology

Cell adhesion first occurs on the surface when scaffold is implanted in vivo. The micro- and nano-structured surfaces have significant effects on cell behavior[8]. Two phase separation methods are used for preparing different micropores: solid-liquid phase separation and liquid-liquid phase separation. Solid-liquid phase separation of the PLLA/HA−dioxane system is the nucleation and growth process of the solvent crystal during freezing. The difference between refrigerator temperature and crystallization point temperature is supercooling. High supercooling leads to small grain which means small pores after solvent extraction. Scaffolds shown in Fig.7a,7b have the same supercooling of dioxane crystallization, so micropore is at same size. The same morphologies between Fig.7c,7d are also observed. But larger supercooling leads to smaller interval between micropores, compared with Fig.7c, 7d and Fig.7a, 7b. So micropore morphology could be controlled by adjusting supercooling.

When water was added in the solvent 1,4-dioxane with ratio 12:88(*v:v*), a liquid-liquid phase separation took place in the extruded slurry. Fig.8 shows the hierarchical porous structure. The micropores are isotropy, well interconnected and at the same size with cell. These features may be better for cell culture than
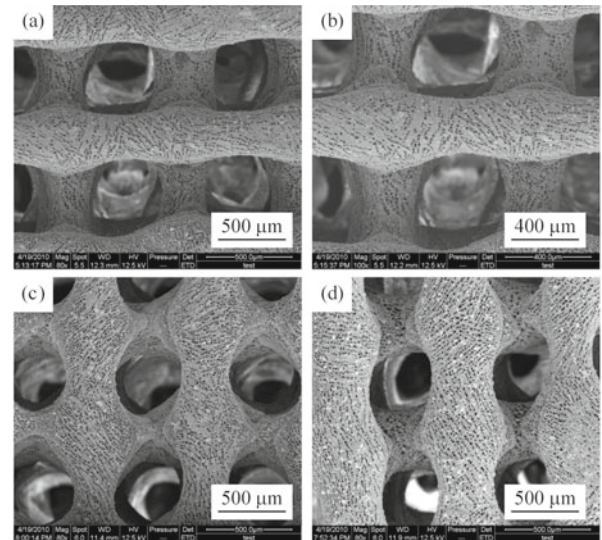
micropores shown in Fig.7.

Fig.7　SEM images of scaffold fabricated under different nozzle/refrigerator temperatures (a)20/−10℃;(b)15/−5℃; (c)30/−15℃; (d)20/−5℃

Fig.8　The effect of water on the micropore morphology of the PLLA/HA composite scaffolds

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3.4 Effect of HA on mechanical property of PLLA/HA composite scaffolds

Fig.9 shows that the compressive modulus of the porous PLLA/HA composite scaffolds varied with different HA content and different test environment. All scaffolds were fabricated with nozzle temperature 30 ℃ and refrigerator temperature −10 ℃. The concentration of PLLA was constant 8%(wt/v). The compressive modulus increased with HA addition. But the difference between three contents samples in PBS mechanical test condition was not significant as dry condition. A possibility was that the interface of HA particles with PLLA was influenced by PBS.
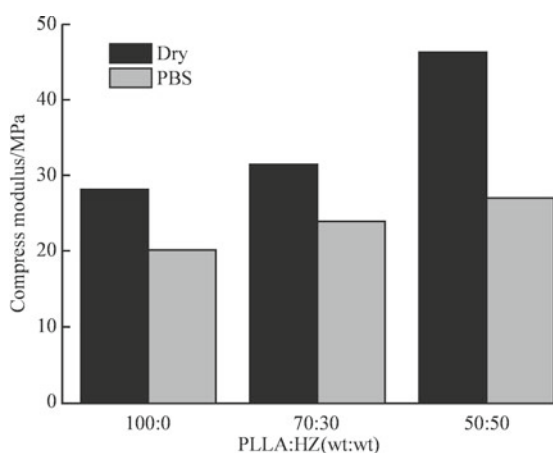

Fig.9 The effect of HA content (wt%) on the compressive modulus of composite scaffolds

3.5 Cell culture

A preliminary biological evaluation was done with pre-osteoblastic cells MC3T3-E1 cultured in the scaffold. The center of scaffold was observed with SEM after cultured for 14 days (Fig.10). The cells grow well and have a good adhesion on scaffold surface. In many research before, new tissue matrix was formed only at the surface layer of the scaffold with only a few cells located in the center[9]. The osteoconductive of nano-HA provides bone cells excellent extracellular matrix and large pores allow for proper diffusion of nutrients in or waste out through the porous scaffold.
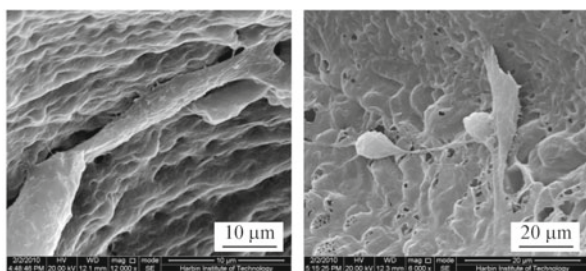

Fig.10 SEM images of MC3T3-E1 osteoblast cells migrated in the center of PLLA-HA scaffolds

4 Conclusion

Porous PLLA/HA scaffolds were fabricated by low-temperature deposition manufacturing. A new precision syringe extrusion nozzle based on ball-screw transmission was developed for fabricating scaffold with large pore of 200-500 μm. With temperature of freezing chamber fixed, thread of polymer solution will disconnect if temperature of material extrusion is too high. On the other hand, if temperature of material extrusion or refrigerator temperature is excessively low, layers will not bond to each other well. Solid-liquid and liquid-liquid phase separation methods are used for preparing different micropores about 10 μm. PLLA/HA with ratio of 50:50(wt:wt) have a highest compressive modulus. Pre-osteoblastic cells MC3T3-E1 migrates into the center of scaffold and a good adhesion occurs after 14 days culture. The composite material scaffold shows good biocompatible and good mechanical property which will be an excellent artificial extracellular matrix for bone tissue engineering. Further biology evaluation between cell and scaffold is ongoing in our lab.

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