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Nano-hydroxyapatite/poly ε-caprolactone composite 3D scaffolds for mastoid obliteration

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Abstract. The aim of this study is to evaluate the use of our nano-HA/PCL composite 3D scaffolds as graft materials for mastoid cavity obliteration in an animal model. Nano-HA particles were synthesized by chemical precipitation technique and mixed them with PCL solution to make composite paste. 3D scaffolds were fabricated by a paste extruding deposition process. The nano-HA/PCL 3D scaffolds showed good in vivo bone regeneration behaviour in a rabbit model after 4 and 8 week implantation. To characterize the 3D scaffolds as a grafting material for mastoid obliteration, mastoid cavities were introduced in rats and implanted the scaffolds. After two week implantation, histological examination showed good tissue ingrowth and new bone formation behaviour. It can be argued that our nano-HA/PCL composite 3D scaffold is a promising alternative material for mastoid obliteration.

1. Introduction

Scaffolds for tissue engineering should be highly porous, tissue supportable and biodegradable. Furthermore, scaffolds for bone regeneration should be osteoinductive as well as osteoconductive. Hydroxyapatite (HA) and poly ε-caprolactone (PCL) composites have been paid much attention as one of the prospective bone scaffold materials, due to their good combination of biodegradability, osteoconductivity and osteoinductivity [1]. In addition to materials, pore structure of the scaffolds is very important to lead cell or tissue in-growth. There are several conventional methods for fabricating porous scaffolds such as particulate leaching, freeze drying, electrospinning and so on [2-4]. However, if the scaffold size is large, it is difficult to obtain three dimensionally interconnected pores for cell or

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tissue in-growth by the conventional scaffolding processes. Further, the conventional techniques have some limitations in reproducibility and three dimensionally complex shaping. Recently layer manufacturing technology has been paid much attention to fabricate porous scaffolds for tissue engineering, because it can provide different pore structure designs with three dimensionally interconnected pores, and it makes on-demand shaping possible by computer aided processes. There have been suggested several methods for fabricating scaffolds, such as fused deposition modeling, selective laser sintering, 3D printing™, multiphase jet solidification, and 3D plotting [5–7]. Among those methods, a modified fused deposition modeling is a preferred process today. We have also recently developed a paste extruding deposition process to fabricate three dimensional (3D) scaffolds. Using this process, we have developed nano-crystalline HA/PCL composite 3D scaffolds for bone regeneration [8].

By the way, the surgical treatment of serious otitis media often requires canal wall down mastoidectomy to ensure complete removal of decease. This otologic surgery yields mastoid cavity which should be eventually obliterated. Different types of biomaterials have been so far used for mastoid obliteration. However, no single graft material has been proved to be ideal [9-11]. The aim of this study is to characterize the in-vivo bone regeneration behaviour of nano-HA/PCL composite 3D scaffold as a candidate graft material for mastoid cavity obliteration. This study discusses the structure of nano-HA/PCL composite 3D scaffolds and the results of bony implantation in a rabbit and rat model.

2. Materials and method

2.1. Synthesis of nano-HA
Nano-hydroxyapatite (nano-HA) was synthesized by a precipitation method using calcium nitrate (Ca(NO3)2·4H2O, Junsei Chemical) and ammonium phosphate ((NH4)2HPO4, Junsei Chemical). Calcium nitrate and ammonium phosphate were dissolved separately in de-ionized water. Calcium nitrate solution was dropped very slowly into the ammonium phosphate solution while stirring and heating at 70°C. The pH of the solution was maintained between 10 and 12 by additions of ammonium hydroxide according to the following equation (1):

$$10\text{Ca(NO}_3\text{)}_2 + 6(\text{NH}_4\text{)}_2\text{HPO}_4 + 2\text{NH}_4\text{OH} = \text{Ca(PO}_4\text{)}_6(\text{OH})_2 + 20\text{NH}_4\text{NO}_3$$  \hspace{1cm} (1)

After titration and reaction were finished, the mixture was aged at room temperature for 24 h to precipitate apatite particles. The apatite precipitates were rinsed more than five times with de-ionized water and treated in de-ionized water for 4 h at 90–100°C. After treatment, the nano-crystalline apatite precipitates were obtained in slurry. This slurry and dimethylformamide (DMF) were mixed in a beaker with stirring, and the mixture was gradually heated up to 100–120°C. After complete evaporation of water, nano-HA/DMF slurry was obtained. The slurry was finally vacuum-dried at room temperature to obtain nano-HA powders.

2.2. Fabrication of nano-HA/PCL 3D scaffolds
Poly ε-caprolactone (PCL) (Sigma Aldrich, Mw 65,000) was dissolved in chloroform at 40 °C to prepare PCL solution. The nano-HA was mixed with PCL solution to make nano-HA/PCL composites. The composite paste extruded out of a syringe and stacked layer by layer to fabricate 3D scaffolds with a rectangular lattice shape using a robotic layer manufacturing machine. The layer manufactured scaffolds were rinsed in distilled water for at least 48 hours and freeze-dried for 72 hours to remove residual solvent completely. The structure of nano-HA/PCL scaffolds were examined by scanning electron microscopy. The porosity of 3D scaffolds was measured using mercury intrusion porosimetry (Auto-pore IV 9500, Micromeritics Instruments, Norcross, USA).
2.3. In-vivo evaluation
New Zealand white rabbits were anesthetized with ketamine hydrochloride and 2% xylazine hydrochloride. A 15 mm-defect was introduced in the tibia of each rabbit. The n-HA/PCL 3D scaffold was implanted in the defect and fixed with a titanium bone plate. The rabbits were sacrificed at 4 and 8 weeks after operation. Female rats were anesthetized with 50 mg/ml of ketamine and 25 mg/ml of rompun. A 3 mm-defect was introduced in the right mastoid of each rat and mastoid mucosa was removed with 2% trichloroacetic acid. Mastoid obliteration was conducted by implantation of n-HA/PCL 3D scaffolds. The rats were sacrificed at 2 weeks after operation. Retrieved samples were fixed in 10% neutral formalin followed by decalcification and paraffin-embedding. The samples were examined radiologically using a high resolution micro-computed tomography system (CT 40, Scanco Medical, Bassersdorf, Switzerland). The scanner was set to a voltage of 55 kV and a current of 145 mA to allow sufficient energy. The samples were scanned at 8-µm voxel (3D pixel) resolution with an integration time of 120 ms to produce reconstructed 3D images. Samples for histological examination were prepared by sectioning the paraffin-embedded samples and staining with hematoxylin and eosin.

3. Results and discussion

3.1. Structure of nano-HA/PCL 3D scaffolds
Fig. 1 shows the morphology, pore and surface structure of a nano-HA/PCL 3D scaffold fabricated by a paste extruding deposition process. The morphology of the scaffold was rectangular lattice shape with a regular array of extruded wires having a diameter of 0.4 mm and a road gap of 1 mm. The views of the scaffold in the x, y, z directions were three dimensionally interconnected pore channels with good regularity. Fig. 1(c) shows that nano-HA particles are exposed and dispersed well on the surface of scaffold. Concerning that the size of synthesized nano-HA particles is ranging from 50 to 100 nm, no big agglomeration was found. We have confirmed that these well dispersed nano-HA particles are very effective to enhance cell attachment and osteogenetic behavior owing to those hydrophilic characteristics and chemical stimulus, respectively [8]. The macro porosity of the scaffold was about 73 vol.%.  

![Fig. 1. The morphology of nano-HA/PCL 3D scaffold(a), and its pore structure(b) and surface(c).](image)

3.2. Micro-CT images
Micro-CT images were taken from the bony tissue formed in the nano-HA/PCL 3D scaffolds after 4 and 8 week implantation in rabbit tibias, as shown in Fig. 2. After 4 week implantation, micro-CT images showed that all pores in 3D scaffolds are uniformly filled with bone-like tissues (brown color). This means that 3D scaffold structure is good for tissue in-growth. After 8 week implantation, the images were somehow dissimilar to 4 week. Brown colored region was decreased, while bright colored region was increased. The bright contrast in micro-CT images may be caused by higher density of materials. Therefore, increase in bright region is believed that mineralization was
progressed in the scaffold. The bright contrast was clearer in the vicinity of host bone than in the center part of scaffolds. However, these micro-CT images can not provide us what kinds of tissue correspond to brown color and bright image in detail.

Fig. 2. Micro-CT images of 3D scaffolds and tissues taken from the rabbit tibias after 4 week (a, b) and 8 week (c, d) implantation. (a, c) and (b, d) correspond to longitudinal and transverse section, respectively.

3.3. Histological views
Histological examination provided us more clear information about tissue regeneration in vivo. Fig. 3 shows histological views of H&E stained tissues with the nano-HA/PCL 3D scaffolds after 4 and 8 week implantation in rabbit tibias. Likewise with micro-CT observation, all pore spaces in 3D scaffolds are filled with bone tissues. This indicates that three dimensionally interconnected pore structure is very important to lead in vivo tissue in-growth. There was no severe inflammatory reaction or adverse tissue reaction in any of the implanted scaffolds. At 4 week implantation (Fig. 3 (a, b)), immature bone tissue (osteoid) was found in the center of scaffold as well as in the vicinity of host bone. A large number of osteoblasts were evident around the newly formed osteoid matrix. At 8 week implantation (Fig. 3 (c, d)), on the contrary, mineralized new bone tissue was found in the center of scaffolds as well as in the vicinity of host bone, and a lot of osteocytes and osteoblasts were found in the newly formed bone matrix. This means that three dimensionally interconnected pore structure facilitates micro- or macro-vascularization into the scaffold, followed by callus and new bone formation. Besides pore structure, of course, it is believed that nano-HA material must have also contributed to osteogenetic procedure. Therefore, it can be argued that nano-HA/PCL 3D scaffolds are really effective for in vivo bone regeneration, owing to three dimensionally interconnected pore
structure and osteoinductive biomaterials. Some sporadic fatty marrow cells and fat-like tissues were observed around and inside the scaffolds. This is interpreted as a large part of scaffold was placed in the bone marrow cavity of rabbit tibia.

Fig. 3. Histological views of H&E stained tissues with 3D scaffolds after 4 week (a, b) and 8 week (c, d) implantation in rabbit tibia. (a, c) and (b, d) are the vicinity of host bone and the interior of scaffold, respectively.

3.4. Use of 3D scaffolds for mastoid obliteration
As aforementioned, mastoidectomy surgery yields large cavity corresponding to a critical size defect, which can not be regenerated naturally in the entire life. Therefore, the cavity should be filled with suitable filler materials for bone regeneration and obliteration. We tried to use our nano-HA/PCL 3D scaffolds for mastoid obliteration with an animal model. Fig. 4 shows histological views of H&E stained tissues with nano-HA/PCL 3D scaffolds after 2 week implantation in the mastoid cavity of rats. It is noted that immature bone tissue (osteoid) was already found in the center of cavity as well as in the vicinity of host bone even after 2 week implantation. It can be therefore argued that the nano-HA/PCL 3D scaffold could be a promising filler material for mastoid obliteration.
Fig. 4. Histological views of H&E stained tissues with 3D scaffolds after 2 week implantation in rats.

4. Conclusion

Nano-HA/PCL composite 3D scaffolds showed good bone regeneration behavior in animal models, owing to three dimensionally interconnected pore structure and osteoinductive biomaterials. The nano-HA/PCL 3D scaffold could be a promising candidate material for mastoid obliteration in otologic surgery.

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