In vitro and *in vivo* biocompatibility of nano-hydroxyapatite/polyamide 66 as bone repair material***

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Abstract

BACKGROUND: Nanohydroxyapatite reinforced polyamide 66 (n-HA/PA66) prepared using the novel process based on nanohydroxyapatite (n-HA) sol may promote the biocompatibility due to the well distribution of n-HA in the polyamide66 (PA66) matrix and chemical bond at organic-inorganic interface. n-HA/PA66 composite has been proposed as a promising bone repair biomaterial.

OBJECTIVE: To investigate the biocompatibility of the novel material both *in vitro* and *in vivo*.

METHODS: Primarily cultured osteoblasts were co-cultured with n-HA/PA66 and PA66. The cell attachment and morphology were studied using phase contrast light microscope (PCLM) and field-emission scanning electron microscope (FE-SEM). Moreover, n-HA/PA66 pins, with PA66 pins as control, were implanted into the right and left (control group) shinbone shafts of the rabbits, respectively. Materials were harvested at weeks 2, 8 and tissue sections were observed.

RESULTS AND CONCLUSION: n-HA/PA66 and PA66 exhibited no cytotoxicity with osteoblasts, while the cellular morphology near the samples was better in the test group than in the control one. Moreover, the cell density attached on n-HA/PA66 was higher than that on PA66 and the cell number were especially different after co-culturing for 3 days ($P < 0.01$). The osteoblasts at the interface between the host bone and n-HA/PA66 also had a higher activity during the earlier period after implantation. Meanwhile the bone formation process was faster and effective in the experimental group. Results suggested that n-HA/PA66 prepared base on the n-HA sol has a better biocompatibility as compared with PA66.

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INTRODUCTION

Biotic bones are nanocomposites mainly composed of nano-hydroxyapatite (n-HA) and collagen, and they can be described as a natural composite composed of an organic matrix strengthened by an inorganic calcium phosphate (CaP) phase [1]. As a natural bone composition, hydroxyapatite (HA) is one of ideal materials for bone substitutions due to its intrinsic biocompatibility. However, the limitations such as brittleness and low strength have hindered its use in high-loading applications [2-4]. Polymer has a better flexibility and a similar elastic modulus to the natural bone though has a shortage of bioactivity. From biomimetic point of view, novel n-HA/polymer composites with better mechanical properties and high exposure of the bioactive ceramics to the implant surface are developed [5]. These composites can overcome the limitation of each single material, so they will be better used for efficient clinic needs [6-7]. Polyamide 66 (PA66) has been widely used in clinical application due to its biosecurity. The polar amido bonds form hydrogen bonds between the PA66 molecules, which are similar to the combination of collagen fiber in natural bone. Li *et al* [8-9] prepared n-HA reinforced PA66 (n-HA/PA66) as a bone tissue replacement material and studied on the biomimetic properties of n-HA/PA66. Previous works mainly focused on the mechanical properties and bioactivity in a simulating bodily environment, but less on the interaction between the implant and the tissue. In our lab, we had prepared n-HA/PA66 composite as a bone repair material using a novel process [10]. The morphology, structure and component analysis of the composite had also proved that it would own a good property due to the uniform distribution of n-HA in PA66 matrix. Present study was designed to investigate the biocompatibility of the novel material both in vitro and in vivo and evaluate whether the composite prepared by this new method is safe to use as a biomaterial.

MATERIALS AND METHODS

Design

Contrast observation.

Time and setting

The experiments were performed at the School of Materials Science and Engineering, Tianjin University of Technology, and Affiliated Hospital of Medical College of the Chinese People's Armed Police Forces between December 2007 and March 2009.

Materials

Experiment animals

A total of 10 neonatal (24 hours) Sprague-Dawley rats were provided by the Radio-medicine Institute of Chinese Academy of Medical Sciences, and 12 adult New Zealand rabbits of 2.5-3.0 kg in weight were provided by the Animal Experimental Center of the Academy of Military Medical Science of Chinese PLA. Protocols were conducted in accordance with Animal Ethics.

Main reagents and equipment used in this study

Reagent and equipment	Source
Dulbecco's Modified Eagle's Medium (DMEM), collagenase type I, trypsin, ethylenediamine tetraacetic acid (EDTA)	GibcoBRL, USA
Fetal bovine serum	Hyclone, USA
TG-150 CO_2 Cell Incubator	Jouan, France

Methods

Material preparation

Nano-hydroxyapatite/polyamide66 (n-HA/PA66) composite powder was synthesized in our laboratory [10]. Briefly, PA66 (Asahi Chemical Industry Co. Ltd., Japan) with a viscosity-average molecular weight of 18 kDa was completely dissolved in formic acid. Then the PA66 and formic acid solution was gradually added into a stable sol of n-HA, with vigorously stirring for 2.5 hours at 90 ℃. The mixture was centrifuged for 10 minutes at 10 000 r/min. After totally washed by deionized water six times, n-HA/PA66 composite powder was obtained after being dried for 24 hours at 65 ℃. The machine for injection molding (SY300-Ⅲ, China) was used to form different samples at 220 ℃.

The samples for the cell culture tests were processed into rectangular pieces with a size of 8 mm×8 mm×3 mm. The samples for the animal surgery were processed into pins with a size of Φ2 mm×6 mm. For comparison, the materials used in this study were PA66. All samples were ultrasonically rinsed in aceton, distilled water, absolute ethyl alcohol, distilled water in turn, and then air-dried. Samples were autoclaved at 126 ℃ for 40 minutes prior to the tests.

Osteoblast isolation and culture

Osteoblasts were isolated from the neonatal Sprague-Dawley rat calvarias. The calvarias were isolated, and all connective tissues were carefully removed and then carefully washed by DMEM. The parietal bones were minced into pieces measuring about 1 mm× 1 mm using sterile surgical scissors. Osteoblasts were isolated by an enzyme solution containing collagenase type I and DMEM. Following 10 minutes of incubation, the released cells were discarded to prevent contamination with other cell types. The minced bones were redigested with the enzyme solution for 20 minutes, and then the cell suspension was kept at 4 ℃. The process was repeated twice, and then finally the collected solution was centrifuged for 10 minutes at 1 000 r/min. Cells were plated into tissue culture flasks with culture medium, DMEM supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, 100 μ/mL penicillin and cultured in a humidified incubator at 37 ℃ with 5% CO_2. The primary osteoblasts were then left until confluence was reached.

Co-culture

Cells of the second passage were used for this study. Samples were separately placed in the bottom of 24-well flat-bottomed cell culture plates. Cells suspensions (3.2×10^7 cells/L) were gently placed on each sample. The plates were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2. PCLM (TE2000-U, Nikon, Japan) was used to observe the morphology of living cells at the edge of the samples every day. After incubation at different time points, the unattached cells in the wells were removed using a phosphate buffer saline (PBS) solution and the attached cells were fixed with 3% glutaraldehyde solution for cellular morphology observations. The gold coated samples were observed by FE-SEM (JOEL, 6700F, Japan). For the proliferation tests, after co-culturing for 1, 3, 5 and 7 days, cells were detached in 0.25% trypsin-EDTA solution and the number of cells in each well was determined with a hemocytocounter.

Surgical procedure

A total of 12 adult New Zealand rabbits were randomly assigned to four groups, in which each rabbit was implanted with three PA66 pins into the left shinbone shaft and three n-HA/PA66 pins into the right shinbone shaft. Animals were generally anesthetized for surgery and the shinbone region was scrubbed with 25 g/L tincture of iodine and 75% ethanol. The pins were screwed into the rabbit's shinbone shaft after pre-drilling with a 2 mm hand-operated drill. All animals received a subcutaneous injection of gentamicin sulphate. Each group of rabbits was sacrificed 2 and 8 weeks after surgery.

Histological evaluation

Bone samples around the pins were fixed in 10% formaldehyde solution. After being dehydrated and decalcified, the bone samples were embedded in paraffin. The histological evaluation was performed on hematoxylin and eosin stained section by light microscopy (Olympus BX51).

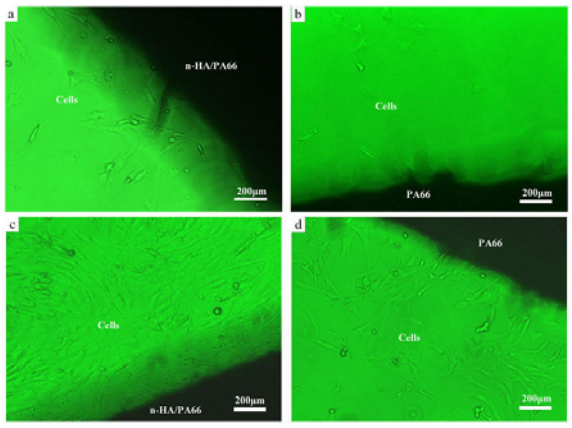
Statistical analysis

Means in each group were analyzed with SPSS 15.0 software. The analysis of independent-sample *t*-test was used to evaluated the significant differences between the results.

RESULTS

In vitro evaluation

Figures 1a, b show the micrographs of osteoblasts cultured for 1 day near the samples of n-HA/PA66 and PA66, respectively. As shown in Figure 1a, the osteoblasts near the n-HA/PA66 sample exhibited the normal individual form of the cells with the shape of shuttle and/or elongated polygon. Cell morphology near both of the samples was similar, but the number of attached cells to PA66 (Figure 1b) was slightly lower than that on n-HA/PA66. After 5-day culture, the cells near the edge of the n-HA/PA66 and PA66 specimens are shown in Figures 1c, d. The osteoblasts attached very well to the n-HA/PA66 and PA66 with normal shape, but the cell density near the n-HA/PA66 was higher than that near the PA66. In addition, the osteoblasts were in more close contact with each other and apoptotic cells were observed near the n-HA/PA66, which was regarded as the normal physiological processes.



Black areas at the corners of the photographs are the borders and shadows of the test samples (×100)

Figure 1 Light micrographs of osteoblasts cultured for 1 day on nanohydroxyapatite reinforced polyamide 66 (n-HA/PA66) (a), PA66 (b) and 5 days on n-HA/PA66 (c) and PA66 (d)

As shown in Figure 2, the morphology of the osteoblasts cultured for 1 and 5 days on n-HA/PA66 and PA66. The different responses of the cells to the different samples were obvious. The cells on the surface of the PA66 (Figures 2b, d) maintained a round or spindle-like morphology during the whole incubation period. For the n-HA/PA66, the cells were sail-like, elongated and thicker in the central area of the nucleus and nucleolus and flattened in the peripheral regions after 1-day incubation. Some cells spread across the surface and contacted with each other (Figure 2a). After a 5-day culture, the cells and the excreted matrixes were connected together and it was hard to distinguish between the cells and the matrixes (Figure 2c). Obviously, n-HA/PA66 showed significantly better cell response than PA66 during the whole incubation period. In addition, the cells on the surface of the composite excreted more matrixes than the cells on the surface of PA66.

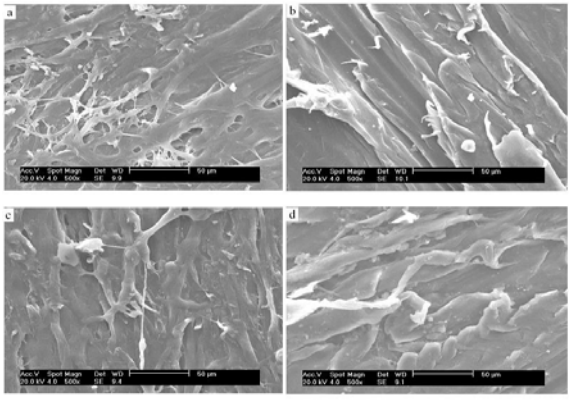


Figure 2 Scanning electron micrographs of osteoblasts cultured for 1 day on nanohydroxyapatite reinforced polyamide 66 (n-HA/PA66) (a), PA66 (b) and 5 days on n-HA/PA66 (c) and PA66 (d)

As shown in Figure 3, the cell growth of osteoblasts on the surfaces of each group specimens were evaluated with cell density after culturing for 1, 3, 5 and 7 days. The mean cell number per area increased during the incubation for both n-HA/PA66 and PA66 specimens. The cell density of osteoblasts was higher on the n-HA/PA66 as compared to the PA66 at all designated incubation time. Meanwhile, there were statistical differences in the mean unit cell number for the two specimens after culturing for 1 and 3 days ($P < 0.05$), especially 3 days ($P < 0.01$).

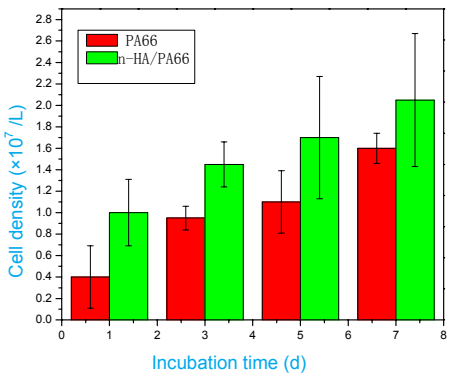
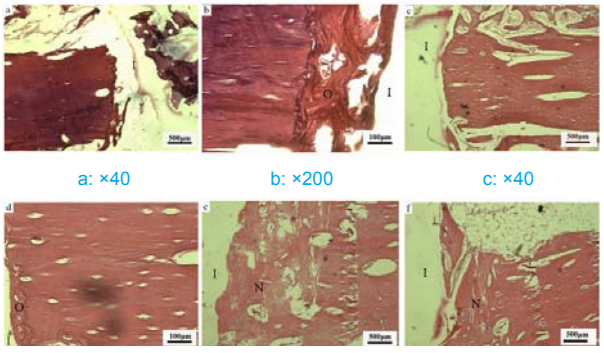


Figure 3 Cell density of osteoblasts cultured on nanohydroxyapatite reinforced polyamide 66 (n-HA/PA66) and PA66 samples for various time points

Histological findings

Figure 4 shows the optical microstructure of the interfaces between the n-HA/PA66 and PA66 implants and new bone after 2 and 8 weeks postimplantation. No inflammatory signs or adverse tissue reaction were observed. During the earlier period post implantation, there was a band of newly formed osteoid tissue between both the implants and the host bone (Figures 4a–d), and several osteocytes embedded in the band area, as indicated by “O” in Figures 4b, d. The formed new osteoid tissue was thicker and connected more closely to the n-HA/PA66 compared with the PA66.

After 8 weeks, the formation of new bone was clearly visible, as indicated by “N” in Figures 4e, f. In the case of the control, the arrangement of bone trabecula was in good order (Figure 4f). In contrast, a much higher osteoinductive activity, which was evident by a large quantity of osteoblasts aligning along the edge of the neoformative bone were seen in the n-HA/PA66 group. Bone trabeculaes connected together and covered the implant surface completely with mature osteocytes embedded (Figure 4e).



a: ×40 b: ×200 c: ×40

d: ×200 e: ×40 f: ×40

I: implant; O: osteoid tissue band; N: newly formed osteoid tissue

Figure 4 Pathological photographs of the implant/bone interfaces after 2 (a–d) and 8 (e, f) weeks postimplantation (Hematoxylin-eosin staining)/(a) (b) (e) nanohydroxyapatite reinforced polyamide 66 (n-HA/PA66), (c) (d) (f) PA66

DISCUSSION

Cell culture method is a fast and sensitive method to qualify the toxic effects of the biomaterials *in vitro*. In this study, the *in vitro* responses of osteoblasts to n-HA/PA66 and PA66 were investigated to confirm the basic biocompatibility of them. They showed no obvious cytotoxicity for the time period investigated. From the comparison point of view, the n-HA/PA66 samples exhibited better cytocompatibility than the PA66 samples. The results agree with much of the work previously published on n-HA/PA66 in that the biocompabibility of n-HA/pA66 is promising and approximates that of PA66 [11]. The present *in vitro* test results demonstrated that the rat osteoblasts exhibited better growth on the surfaces of n-HA/PA66 samples than in the control ones. The existence of HA nano-particles might contribute to the increased cell viability [12].

The result of *in vitro* experiments prompted us to study the materials *in vivo*. The direct bone contact was found in the interfaces of host bone and both n-HA/PA66 and PA66. The results from histology indicated that PA66 has good

biocompatibility and osteoconductivity. In comparison, n-HA/PA66 presented not only good biocompatibility but also faster and more effective bone formative activity at the implant area. The Ca^{2+} and PO_4^{3-} ion-exchange reaction between the n-HA and surrounding body fluids results in the formation of an apatite layer on the implant. The bone healing process is therefore enhanced by this biological apatite layer. Osteoblasts play an important role during the bone formation process. They excrete not only osteoid which then calcified into bone matrix but also a variety of regulatory factors. These regulatory factors have an effect on regulating both bone formation and bone resorption, and promoting bone calcification. The n-HA particles dispersed on the surface of the implant have a strong adhesion to osteoblasts[13], which may influence the capacity of these cells to proliferate and to differentiate themselves upon contact with the implant. The strong adhesion of n-HA to osteoblasts also weakens adhesive function of fibroblasts and competes against cells the proliferation of which leads to the undesirable formation of connective tissue and implant rejection. As the results of Figure 4a, b shown, there was not obvious fibroblast adhesion observed at the interface 2 weeks post implantation. Moreover, the nHA/PA66 also showed high osteoinductive activity with a large quantity of osteoblasts aligning along the edge of the neoformative bone 8 weeks postoperation (Figures 4e). The bone formation is also a dynamic process involving osteoblasts and osteocalsts cooperation. Frankenburg *et al*[14] clarified the mechanism HA degraded by osteoclasts and Webster *et al*[15] proved that osteoclast-like cells had a high activity on nanophase ceramics. The high activity and the favorable hydrophilic property of nHA are propitious to the relative protein adsorption. These may probably promote the interaction between the osteoclasts and nHA/PA66 during the bone formation process.

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骨修复用纳米羟基磷灰石/聚酰胺 66 的体内外生物相容性***★

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摘要

背景：采用基于纳米羟基磷灰石溶胶新方法制备纳米羟基磷灰石/聚酰胺 66 复合材料，该材料提高了纳米羟基磷灰石在聚酰胺 66 基体中的均匀分布和二者的有效键合，进而有利于改善材料的生物性能，有望成为新型骨修复材料。

目的：评价纳米羟基磷灰石/聚酰胺 66 复合材料体内外生物相容性。

方法：①将原代培养的成骨细胞与纳米羟基磷灰石/聚酰胺 66 及聚酰胺 66 材料复合培养，使用倒置相差显微镜和场发射扫描电子显微镜观察材料周围及表面的细胞形态。②将纳米羟基磷灰石/聚酰胺 66 复合材料植入兔右侧胫骨，将聚酰胺 66 作为对照组材料植入兔左侧胫骨。在术后 2，8 周，取材料周围骨组织进行病理组织切片观察。

结果与结论：①纳米羟基磷灰石/聚酰胺 66 和聚酰胺 66 未表现出明显的细胞毒性，纳米羟基磷灰石/聚酰胺 66 材料周围细胞形态好于聚酰胺 66，且纳米羟基磷灰石/聚酰胺 66 表面细胞数量多于聚酰胺 66，在复合培养的第 3 天差异尤其显著($P < 0.01$)。②在植入早期，与纳米羟基磷灰石/聚酰胺 66 相接的骨组织成骨细胞活跃且该组材料周围的骨形成过程较对照组更快。结果说明纳米羟基磷灰石/聚酰胺 66 复合材料较聚酰胺 66 有更好的生物相容性。

关键词：纳米羟基磷灰石/聚酰胺 66；聚酰胺 66；生物相容性；细胞培养；骨修复材料

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