Preparation and characterization of nano-hydroxyapatite/chitosan composite scaffolds

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Abstract: A novel nano-hydroxyapatite (HA)/chitosan composite scaffold with high porosity was developed. The nano-HA particles were made in situ through a chemical method and dispersed well on the porous scaffold. They bound to the chitosan scaffolds very well. This method prevents the migration of nano-HA particles into surrounding tissues to a certain extent. The morphologies, components, and biocompatibility of the composite scaffolds were investigated. Scanning electron microscopy, porosity measurement, thermogravimetric analysis, X-ray diffraction, X-ray photoelectron spectroscopy, and Fourier transformed infrared spectroscopy were used to analyze the physical and chemical properties of the composite scaffolds. The biocompatibility was assessed by examining the proliferation and morphology of MC 3T3-E1 cells seeded on the scaffolds. The composite scaffolds showed better biocompatibility than pure chitosan scaffolds. The results suggest that the newly developed nano-HA/chitosan composite scaffolds may serve as a good three-dimensional substrate for cell attachment and migration in bone tissue engineering. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 75A: 275–282, 2005

Key words: biocompatibility; chitosan; hydroxyapatite; nano; scaffold

INTRODUCTION

The regeneration of bone is one of the major difficulties in clinical surgery because many conditions including trauma, tumor, and bone diseases such as osteitis and osteomyelitis can cause bone defects. To restore the structure and function of bone, many solutions have been used in therapy and research, including autografts, allografts, xenografts, and other artificial substitutes.1–3 However, these solutions are not perfect and each has its specific problems. Autografts need secondary surgery to procure donor bone from the patient’s own body and the amount of donor bone is limited.4,5 Allogenic bone substitutes and xenografts bear the risk of infections and immune response.6 Artificial substitutes such as metals and bioceramics do not behave like true bone and they are hard to inosculate with surrounding tissues. Tissue engineering substitutes are another choice for treating bone defects. Recently, three-dimensional (3-D) porous scaffolds loaded with specific living cells have been researched in order to regenerate tissue in a natural way.

Hydroxyapatite (HA) is one of the major constituents of the inorganic component in human hard tissues (bones and teeth), and it is one of the most common biomaterials studied in bone tissue engineering because of its good biocompatibility.7,8 It can form a direct chemical bond with surrounding tissues and is osteoconductive, nontoxic, noninflammatory, and nonimmunogenic.9–12 However, there are some limitations in its usage because its brittleness makes it hard to shape. Bone is considered as a nanocomposite of minerals and proteins, and recently nano-level HA has been investigated and demonstrated as having a good impact on cell–biomaterial interaction.13,14 However, the migration of the nano-HA particles from the implanted site into surrounding tissues might cause damage to healthy tissue.15 To find a resolution, composites of nano-HA and polymers were researched to find a material that retained the good properties of nano-HA and prevented the nano-HA particles from migrating.

Chitosan, a polysaccharide and natural polymer, is
the partially deacetylated form of chitin that can be extracted from crustacea. It consists of glucosamine and N-acetylglucosamine units linked through 1–4 glycosidic bonds and thus shares some bioactivities with various glycosaminoglycans and hyaluroic acid present in articular cartilage. In addition, it degrades in the body to nonharmful and nontoxic compounds. A composite of HA and chitosan therefore is expected to be a good biomaterial for bone tissue engineering. Some researchers have investigated a composite of HA combined with chitosan. HA powder was mixed with chitosan solution by full agitation and then the mixture was freeze-dried into porous scaffolds. In this research, a chemical wet method was used to produce HA in situ. It was easier to get a homogeneous mixture of two solution systems than of solid powder and solution. A more homogeneous composite was obtained. Some researchers made a composite of chitosan and nano-HA paste, but it did not have porosity and could not be loaded with cells. In this research, homogenous nano-HA/chitosan composite scaffolds with porous structure were prepared using a lyophilization method, and the micro-structure and physical and chemical properties were studied by using scanning electron microscopy (SEM), porosity measurement, thermogravimetric analysis (TGA), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), and Fourier transformed infrared (FTIR) spectroscopy. The biocompatibility of the composite scaffolds was evaluated by using methylthiazol tetrazolium (MTT) assay and Wright’s staining after seeding with MC 3T3-E1 cells.

MATERIALS AND METHODS

Materials

Chitosan was obtained from Haisheng Co. (Qingdao, China). The degree of deacetylation was estimated to be 93.5% with the $^1$H NMR method and the viscosity molecular weight was $1.8 \times 10^6$ Da. Ca(NO$_3$)$_2$ and (NH$_4$)$_2$HPO$_4$ were of analytic grade and purchased from Beijing Chemical Engineering Factory (Beijing, China).

Methods

Preparation of porous nano-HA/chitosan

A chitosan aqueous solution of 2 wt % was prepared by dissolving chitosan powder into distilled water containing 0.2 mol/L acetic acid. Then, under agitation, 0.1 mol/L Ca(NO$_3$)$_2$ aqueous solution was slowly added into the chitosan solution. Subsequently, 0.06 mol/L (NH$_4$)$_2$HPO$_4$ was added drop by drop. The mixed solution was blended thoroughly and added to a 24-well plate to freeze at $-20^\circ$C. The frozen composites were lyophilized and the dry composite scaffolds were soaked in 10 wt % NaOH solution for 10 h. The scaffolds were rinsed in distilled water to neutrality and lyophilized again. Finally, we obtained the columned scaffolds with 1.4 cm in diameter and 0.7 cm in height.

SEM examination

The scaffolds with and without cells were fixed with 2 vol % formaldehyde/2.5 vol % glutaraldehyde in 0.1 mol/L phosphate-buffered saline (PBS) for 30 min. After being washed with PBS four times, they were dehydrated in graded concentrations of ethanol (30, 50, 70, 90, 95, and 100%). All the samples were lyophilized and cut by a razor blade to expose the inner parts. After coated with gold in a sputtering device, they were examined with a scanning electron microscope (KYKY-2800; Apparatus Factory, Chinese Academy of Sciences, Beijing, China) with an accelerating voltage of 20 kV.

Porosity measurement

The porosity of the scaffolds was measured with a mercury porosimeter (Autopore IV 9500; Micromeritics Instrument Corp., Norcross, GA).

TGA

After being washed with distilled water fully, the composite scaffolds were freeze-dried for the TGA. The composition of the composites was determined with TGA performed in air between 25$^\circ$ and 800$^\circ$C at a heating rate of 20$^\circ$C/min (STA409; Netasch Co., Germany).

XRD

To investigate the components of the composite, the samples were analyzed with an X-ray powder diffractometer (D8 Advance; Bruker, Germany) using a monochromatic Cu K$\alpha$ radiation.

XPS analysis

XPS (PHI-5300 ESCA, Philadelphia, PA) analysis was used to evaluate the chemical state and atomic ratio of the elements in the composite scaffolds.

FTIR

A PerkinElmer system 2000 FTIR spectrometer (PerkinElmer, Norwalk, CT) was used for FTIR analysis. The spectra were collected over the range of 4000–400 cm$^{-1}$.
Cells from cell line MC 3T3-E1 (a clonal preosteoblastic cell line derived from newborn mouse calvaria, which is often used in bone tissue engineering research) were cultured in α-Eagle minimum essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Jianghai Bio Co., China), 100 U/mL penicillin (Sigma, St. Louis, MO), and 100 μg/mL streptomycin (Sigma). Cells were incubated at 37°C in a 5% CO2 incubator and the medium was changed every 2 days. When the cells reached the stage of confluence, they were harvested by trypsinization followed by the addition of fresh culture medium to create a cell suspension. A cell suspension with a concentration of 2 × 10^6 cells/mL was loaded into the 3-D spongy scaffolds, with 200 μL of suspension for each scaffold. The scaffolds were put in a polystyrene 24-well flat-bottom culture plate and incubated at 37°C in a 5% CO2 incubator. After cells attached, about 6 h, fresh culture medium was added until the total medium volume was 1 mL. Culture medium were changed every 2 days.

Cell morphology examination

The SEM process described above and Wright’s stain method were used in the cell morphology examination.

Cell proliferation assessment

A modified MTT assay was applied in this study to quantitatively assess the number of viable cells attached and grown on the tested scaffolds. Briefly, all the tested scaffolds were fetched to a new 24-well flat-bottom culture plate, and 1 mL of serum-free medium and 100 μL of MTT (Sigma) solution (5 mg/mL in PBS) were added to each sample, followed by incubation at 37°C for 4 h for MTT formazan formation. The upper solvent was removed and 1 mL of 10% sodium dodecyl sulfate (Sigma) in 0.01N HCl was added to dissolve the formazan crystals for 6 h at 37°C. During the dissolving period, the spongy scaffolds were squeezed every 30 min to ensure the complete extraction of the formazan.

Figure 1. SEM images of spongy porous scaffolds. Original magnifications were: (a) chitosan scaffolds, 80×, (b) chitosan scaffolds, 500×, (c) composite scaffolds, 80×, and (d) composite scaffolds, 500×.
crystals. The optical density (OD) at 570 nm was determined against the sodium dodecyl sulfate solution blank. Three parallel replicates were read for each sample.

RESULTS AND DISCUSSION

Morphology analysis

The morphology of the composite scaffold was examined with SEM. Both the chitosan-only scaffolds and composite scaffolds showed a spongy appearance (Fig. 1). Both of them had high porosity and good interpore connectivity. The pure chitosan scaffolds and composite scaffolds were similar in their macroscopic morphology, which indicated that adding the HA in the system did not influence the porous structure. However, the microscopic morphology on pore-wall surfaces was quite different. The surface of pure chitosan scaffolds was smooth, and, on the surface of composite scaffolds, many nano-HA particles were scattered like islands (Fig. 2). The size of each HA particle was about 70–100 nm in width and about 140–260 nm in length. There were about 10–50 HA particles in a square micron. The quantity of the HA particles varied with the various amounts of Ca(NO3)2 and (NH4)2HPO4 added before. The HA particles were scattered in the composite homogenously. The HA inlaid on the pore-wall surfaces of the composite scaffolds might increase the biocompatibility of the scaffolds because of the well-known good biocompatibility of HA. The biocompatibility examination will be discussed below. In addition, the porous structure of the scaffolds made it possible to produce a hybrid system with seed cells.

The porosity of the scaffolds was determined with a mercury porosimeter. The porosity of the scaffolds made with the lyophilization method was 95%, and, according to the SEM images, the pore diameter was mainly at about 20–60 μm. Previous research suggested that human osteoblasts can penetrate pores >20 μm in size,20 and the porosity needs to be >30% to achieve interconnection.21 The porosity of the composite scaffolds prepared in this research was sufficient for good interconnection and exchange of nutrition. Also, the pore size of the scaffolds could support the growth of cells. In this

Figure 2. Microscopic morphologic images of the scaffolds, original magnification 10,000×. (a) Chitosan scaffolds, and (b) composite scaffolds.

Figure 3. XRD patterns of HA, chitosan, and composite scaffolds.
research, MC 3T3-E1 cells were seeded on the scaffolds. They could grow into the scaffolds and distribute uniformly in the scaffolds. After 7 days' culture, the cells proliferated and appeared as many clusters of cells. Figure 8(a) shows cells in one of the clusters in the composite scaffolds. They distributed uniformly and spread well. In addition, our laboratory is conducting further investigation on increasing the pore size based on the fact that the pore size of the composite scaffolds could be improved by controlling the temperature and time of prefreezing according to the research of Madihally and Matthew.22

Compositional analysis

The components of the composite scaffolds were analyzed with XRD, XPS, and FTIR. XRD patterns of HA, chitosan, and composite scaffolds are shown in Figure 3. The XRD pattern was verified by the Power Diffraction File (HA: PDF Card No. 090432; chitosan: PDF Card No. 391894). In the composite scaffolds, the broad peak that appeared around 20° was assigned to chitosan (20.305°, 21.290°) and the sharp diffraction characteristic peaks that appeared at around 31.8° and 25.9° corresponded to...
the peaks of HA (31.773°, 25.879°). It indicated that, in the composite scaffolds, HA was formed.

XPS analysis was done to evaluate the surface elements and the ratio of elements (Fig. 4). Ca and P were found on the surface of the composite, and the mol ratio of Ca/P was 1.8:1, higher than the ratio of Ca/P of pure HA, 1.67:1, which means that perhaps some carbonate had been incorporated into the composite. Even if a carbonate-free solution is used, carbonate is able to be incorporated into apatite from the atmosphere. In addition, the results of FTIR also showed the existence of a carbonate group at 1417 cm⁻¹, along with the peaks of phosphate groups and hydroxyls, the functional group of HA (Fig. 5). All the above results suggested that HA might be formed in the composite scaffolds with some carbonate incorporation.

To check whether the composite scaffolds could prevent the nano-HA particles from diffusing, TGA was conducted on the composite scaffolds after they were washed thoroughly with distilled water. From the TGA curves, it was found that, with the temperature increasing up to about 600°C, chitosan scaffolds lost nearly all the weight whereas HA lost little weight. The composite scaffolds lost their weight rapidly with increasing temperature up to 600°C, but in the range of 600°C–800°C, it changed little [Fig. 6(a)]. This indicated that after 600°C nearly all the chitosan in the composite was lost and HA remained. The mineral contents in the synthesized composite scaffolds could be assessed from the TGA data and were a close match to the stoichiometric amounts of the original reactants as shown in Figure 6(b). Therefore, it could be concluded that washing did not remove the nano-HA particles away.

**Biocompatibility examination**

The biocompatibility of the composite scaffolds was assessed on cell proliferation and cells’ morphology. Cell proliferation was examined with MTT assay (Fig. 7). The same amount of MC 3T3-E1 cells were seeded on chitosan scaffolds and composite scaffolds. After 7 days’ culture, the amount of cells on the composite scaffolds was significantly higher than that on the chitosan scaffolds, which indicated that the MC 3T3-E1 cells showed much better proliferation properties on the composite scaffolds than on pure chitosan scaffolds.

To compare the morphology of cells grown on the two scaffolds, cells on the composite scaffolds and chitosan scaffolds were fixed and stained with

![MTT assay of cells grown on chitosan scaffolds and composite porous scaffolds. Data represent the mean ± SD for three samples. *p < 0.01 compared with pure chitosan scaffolds at the same culture time.](image)

![Wright’s stain images of cells grown on the scaffolds, original magnification 200×. (a) Composite scaffolds, and (b) chitosan scaffolds.](image)
Wright’s stain. Shutter-like and polygon-shaped cells grew on the semitransparent composite scaffolds (Fig. 8). The cells on the composite scaffolds [Fig. 8(a)] spread better than those on the chitosan scaffolds [Fig. 8(b)]. In addition, to examine the cell growth in the pores, the cells of composite scaffolds were detected with SEM (Fig. 9). Some cells grew into the pores of scaffolds; they adhered to the pore walls and spread well. Both the cell proliferation assay and cell morphology inspection showed that the combination of nano-HA increased the biocompatibility of the scaffolds significantly.

CONCLUSION

In this research, homogenous composite scaffolds of chitosan and nano-HA were prepared and investigated. Chitosan and HA were combined homogenously through the in situ synthesis of nano-HA using the wet chemical method. The porous structure of the composite scaffolds was made by the lyophilization. The spongy scaffolds showed good porosity and some cells could grow in the pores of these 3-D scaffolds. On the pore walls of the scaffolds, the nano-HA particles were inlaid in the chitosan surface like islands. The composite scaffolds showed better biocompatibility than chitosan scaffolds. Cells grown on the composite scaffolds were in a better state and had a higher proliferation.

The nano-HA particles bonded to the chitosan scaffold tightly and the combination prevented the nano-HA particles from diffusing to a certain extent. It was also easy to shape. As a result, some of the obstacles inherent in the use of HA in tissue engineering might be overcome.

In addition, the porous structure of the scaffolds and good biocompatibility could combine with cells in vitro to form a hybrid system for tissue engineering, which is more useful, and the quality is easy to control. The results indicate that the porous nano-HA/chitosan composite scaffolds are promising materials in tissue engineering research.

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