Preparation of bonelike apatite composite for tissue engineering scaffold

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Abstract

A novel sponge composed of a poly (lactic acid) composite skeleton covered with bonelike apatite (b-HA) was produced via a particle-leaching technique combined with a biomimetic processing. The sponge has a large porosity of \( \approx 75\% \) with large-sized pores and shows mechanical ductility. After incubation of human osteoblasts for 7 days, numerous cells attached to the surface of the skeleton, which was covered with b-HA. One result of the osteoclastic cell culture showed that the b-HA on the composite has excellent bioresorbability. The sponge is expected to be one of the promising candidates for bone tissue engineering scaffolds.

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1. Introduction

While historically, organic tissue has been employed in the repair of bone defects; more recently, attention has been paid to synthetics, which may obviate the problems arising from the use of organic tissue in bone grafting. The use of all allografts carries the risk of disease transmission and tissue rejection, while autografts present problems of limited supply and donor site morbidity. These facts have made synthetic grafts an alternative to organic grafts. Some ceramics, such as Bioglass\textsuperscript{\textsuperscript{\textregistered}}, sintered hydroxyapatite and glass–ceramic A–W, have been shown to spontaneously bond to living bone [1–3]. They are called bioactive materials, and are already in clinical use as important bone-repairing materials. These materials could promote the generation of new bone by acting as a scaffold for osseous growth, but they are only osteoconductive and not osteoinductive.

Vacanti et al. have developed a new technique called 'tissue engineering' [4]. Engineering living tissue for reconstructive surgery requires an appropriate cell source, optimal culture conditions, and a biodegradable scaffold as the basic elements. A scaffolding material is used either to induce formation of bone from the surrounding tissue or to act as a carrier or template for implanted bone cells or other agents. To serve as a scaffold for bone tissue engineering, the material must be biocompatible, osteoconductive, and have a macroporous structure. Calcium phosphate ceramics such as hydroxyapatite or \( \beta \)-tricalcium phosphate (\( \beta \)-TCP), which have osteoconductivity, were reported to have been applied to scaffolds for bone tissue engineering [5,6].

Recently, much attention has been paid to bonelike hydroxycarbonate apatite (b-HA) as a novel biomaterial, since b-HA is very similar to apatite in terms of living bone in its chemical composition and structure [7] and shows effective compatibility in cell attachment, proliferation, and differentiation on the material [8], as well as good bioresorbability [9]. We expect that the sponges composed of b-HA skeleton can be applied to scaffolds for bone tissue engineering.

In general, ceramics show brittleness and low resistance against impact loading. Such ceramic sponge materials have a serious risk of breaking in normal handling during operations. To eliminate this risk, it has been reported that the composites were fabricated using a polymer sponge coated with bioactive materials such as hydroxyapatite or Bioglass\textsuperscript{\textsuperscript{\textregistered}} [10,11].
A preparation method for b-HA using simulated body fluid (SBF), which is a tris-buffer solution with inorganic ion concentrations almost equal to those of human plasma, is called a biomimetic method [12]. This method has an advantage over conventional methods in that materials can be coated with b-HA without heating. Two indispensable conditions needed for the formation of b-HA on materials using SBF are (1) the existence of the surface functional groups that induce nucleation of b-HA and (2) increase in the supersaturation of b-HA in SBF [3,13,14].

Poly(lactic acid) (PLA) with many carboxy groups is one of the promising candidates for supplying inducers for the b-HA nucleation. A carboxy group, which is known to induce b-HA nucleation [13], can be formed by the hydrolyzation of PLA. To increase the supersaturation of b-HA in SBF, a large amount of Ca\(^{2+}\) ions should be dissolved from the materials. Biodegradable calcium carbonate is expected to provide Ca\(^{2+}\) ions for SBF resulting in an increase in the supersaturation of b-HA. It is well known that calcium carbonate has three polymorphs, viz. calcite, aragonite and vaterite. The solubility of vaterite is higher than that of calcite or aragonite [15]. We have already reported that the PLA composites containing vaterite have much higher b-HA-forming ability in SBF than the composites containing calcite or aragonite without vaterite [16]. In our earlier work a pellet of a powder mixture consisting of 25 wt% biodegradable PLA and 75 wt% nano-sized calcium carbonate (vaterite) was reported to form b-HA on its surface even after being soaked for 3 h in SBF [17]. It was suggested that rapid formation of the b-HA originated from the fact that PLA contains carboxy groups that are bonded with Ca\(^{2+}\) ions for the b-HA nucleation, and a large amount of vaterite, which has the ability to effectively increase the supersaturation of the b-HA. We believe that various novel biomaterials can be prepared using PLA and vaterite. Hereafter, the composites are denoted by CCPC (Calcium Carbonate/Poly(lactic acid) Composites).

In the present work, we biomimetically prepared a novel sponge composed of PLA composite skeleton covered with b-HA utilizing CCPC. The present study also involves the evaluation of cell-compatibility on CCPC covered with b-HA for application to tissue engineering scaffolds.

2. Experimental method

2.1. Preparation of vaterite powders

Vaterite was prepared by a carbonation process using methanol [18]. CO\(_2\) gas was blown for 3 h at a flow rate of 300 mL/min into the suspension consisting of 7.0 g of Ca(OH)\(_2\) in 180 mL of methanol at 0 °C in a Pyrex\textsuperscript{®} beaker. The resultant slurry was dried at 70 °C in air, resulting in fine-sized powders. Fig. 1 shows an X-ray diffraction (XRD) pattern and scanning electron microscopy (SEM) photograph of the vaterite powders. The XRD pattern shows that the calcium carbonate powders obtained in the present work consist predominantly of vaterite with small amounts of aragonite and calcite. The SEM photograph shows that secondary particles of ≤500 nm diameter were formed as an agglomeration of primary particles of ~100 nm. The BET surface area was measured to be ~40 m\(^2\)/g.

2.2. Preparation of PLA composites containing vaterite powders

PLA produced by Shimadzu Corp. (LACTY#2012) was used as a matrix phase. PLA with a molecular weight of 160±20 kDa, determined by gel permeation chromatography, was dissolved in methylene chloride at room temperature. The vaterite powders were added to the PLA solution and the mixture was then stirred. The weight ratio of vaterite/PLA was 1/2. We have already reported that CCPC containing ~30% vaterite has excellent mechanical properties, such as a bending strength of ~50 MPa, Young’s modulus of ~5 GPa and ductility, with a high b-HA-forming ability in SBF [19]. The slurry mixture was stirred and cast into a stainless steel die, and then dried in air for solidification. After that, the product in the die was heated at 180 °C and uniaxially hot-pressed under a pressure of 40 MPa. After the heating, the specimen in the die was cooled to room temperature.

Fig. 2(a) shows a photo of CCPC in the present work. The sample was picked up and bent with two fingers. CCPC has high flexibility and can be cut using scissors, as shown in Fig. 2(b).
2.3. Preparation of the CCPC sponge skeleton covered with b-HA

2.0 g of PLA was dissolved in 20 mL of methylene chloride at room temperature. The vaterite powders were added to the PLA solution and the mixture was then stirred to prepare a PLA slurry including the vaterite powders. The weight ratio of vaterite/PLA was 1/2. The sponge was prepared using a conventional particle-leaching technique. Sucrose particles, which were sieved with an opening of from 0.5 to 1.0 mm, were added to the PLA slurry. The weight ratio of CCPC/sucrose was 1/6. The slurry mixture was stirred, cast into a stainless steel die, and subsequently dried in air for solidification. After that, the product in the die was heated at 180°C and uniaxially hot-pressed at this temperature under a pressure of 40 MPa to prepare a CCPC/sucrose composite. After the hot-pressing, the specimen was cut in methanol with a diamond saw. Our strategy for the preparation of the sponge composed of CCPC skeleton coated with b-HA is to leach out the sucrose phase and simultaneously form b-HA on the composite skeleton utilizing SBF (consisting of 2.5 mM of Ca²⁺, 142.0 mM of Na⁺, 1.5 mM of Mg²⁺, 5.0 mM of K⁺, 148.8 mM of Cl⁻, 4.2 mM of HCO₃⁻, 1.0 mM of HPO₄²⁻, and 0.5 mM of SO₄²⁻) that included 50 mM of (CH₃OH)₃CNH₂ and 45.0 mM of HCl at pH 7.4 at 37°C as a solvent. The CCPC/sucrose composite was soaked in SBF for 3 days.

The crystalline phases in the sponge were identified by XRD, and the morphology of the sponge observed by SEM. The pore size distribution of the sponge was measured by mercury porosimetry. The compressive strength of the sponge (7×7×10 mm) was estimated by a compressing test at a loading rate of 1 mm/min.

2.4. Osteoblastic and osteoclastic cell cultures

Human bone marrow stromal cells, obtained by aspiration from the femoral diaphysis of patients were used for this experiment. The cell suspension was cultured in mesenchymal stem cell basal medium (MSCBM, Cambrex) with 10% fetal bovine serum (FBS) supplemented with 20 µg/mL L-glutamine, 1 µg/mL penicillin/streptomycin and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Two millilitres of a cell suspension containing (1×10⁴ cell/mL) was plated on the CCPC sponge skeleton covered with b-HA (5×5×5 mm) and the sponge then incubated at 37°C in 5% CO₂ for 1 week.

Osteoclasts were obtained from the long bones of 1-day-old neonatal rabbits (Japan white), following a reference [20]. A α-modified minimum essential medium (α-MEM, Gibco) supplemented with 15% FBS and antibiotics was used as the plating medium. A cell suspension (100 µL) containing 50–100 multinucleated osteoclasts/100 µL was plated onto disk shaped CCPC (with the composition of CaCO₃/PLA=1/2 in weight ratio) covered with b-HA prepared by soaking in SBF for 2 days (8 mmØ×1 mm) plated in the small wells of microculture plates. A compact of CCPC (with the composition of CaCO₃/PLA=1/2 in weight ratio) was used as a control material to compare with CCPC covered with b-HA. After incubation at 37°C in 5% CO₂ for 90 min, the unattached cells were gently washed off and the substrates were transferred into 35 mm culture medium and then incubated at 37°C in 5% CO₂ for 2 days.

After the culturing period, the samples were fixed with glutaraldehyde in a cacodylate buffer. The cells, after being rinsed several times in the same buffer, were post-fixed in 1% osmium tetroxide and dehydrated through graded ethanols. The samples were freeze-dried with tert-butyl alcohol and coated with osmium. The morphology of osteoblasts and osteoclasts, and the resorption lacunae produced by osteoclasts on the surface of the substrates were observed with SEM.
3. Results and discussion

3.1. CCPC sponge skeleton covered with b-HA

Fig. 3 shows the SEM photographs, the XRD pattern, and the pore size distribution of the sample following 3 days of soaking in SBF. The SEM photograph shows that the sponge has numerous, large pores of 450–580 μm in diameter and large interconnected channels of 70–120 μm. The sponge has continuous open foams with a 3D interpenetrating network of struts and pores, while the surface of the CCPC sponge skeleton is covered with numerous deposits. In Fig. 3(b), it is clear that, although an XRD peak of $2\theta \sim 32^\circ$ resulting from the HA is not clear due to superimposition on the calcium carbonate (aragonite), the peak appears anew following the treatment (in comparison with the XRD pattern of the calcium carbonates in Fig. 1). That is, judged from the XRD pattern and the morphology, the deposits are concluded to be b-HA. The pore-size distribution measured using a mercury porosimeter showed that there exist almost no pores below several tens of micrometers in diameter and the median pore size of the sponge is 125 μm. The porosity was estimated from the measurement to be $\sim 75\%$. When a compact of the powder-mixture consisting of CCPC and sucrose was hot-pressed at 180 °C, the sucrose particles melted (it begins to melt at 160 °C), leading to adjacent particles connecting to each other. As a result, both the sucrose and CCPC phases were unified into an interconnected three-dimensional network. The macroporous structure of the sponge may allow the migration of cells into the interior of the sponge.

Fig. 4 shows typical stress–strain curves measured by a compressive tester for the CCPC sponge and $\beta$-TCP sponge with the same porosity of 75%. The CCPC sponge shows the compressive strength of $\sim 1.5$ MPa and the maximum strain for the fracture of the sponge is over 10%. The curve of the CCPC sponge also shows that the fracture proceeds gradually beyond the maximum stress; the sponge leads to ductile fracture. On the other hand, although the $\beta$-TCP sponge has strength close to that of the CCPC sponge, the stress–strain curve shows a typically brittle fracture. The CCPC sponge in the present work will not break in normal handling during operations. This ductility is believed to be an important mechanical property for the tissue engineering.

Fig. 3. (a) SEM photographs of the cut surface, (b) the XRD pattern, and (c) the pore size distribution of the sponge prepared in the present work. (□) aragonite, (△) PLA, and (⊥) apatite. Inset shows the magnified image of the skeleton surface.
3.2. Cells culture on CCPC

Fig. 5 shows a cross-sectional SEM photograph of the skeleton surface around the center of the CCPC sponge following one-week incubation. Numerous cells were attached to the skeleton covered with b-HA. We believe that cells can migrate through the channels into the interior of the sponge. SEM observation showed that the adhesion of cells on the skeleton covered with b-HA is larger than that on the sponge skeleton composed of PLA. This suggests that the presence of the b-HA layer on CCPC induces an effective increase in the attachment of the cells.

Fig. 6 shows SEM photographs of the osteoclast on the surface of CCPC covered with b-HA and CCPC after incubation for 2 days. The resorption lacuna was evident on the surface of these substrates. This area was widespread with a diameter of more than 25 μm on CCPC covered with b-HA. The attacks (∼15 μm of diameter) on CCPC are smaller than that on CCPC with b-HA. Although further statistical analysis concerning the difference between the lacuna sizes of the samples is needed, Fig. 6 clearly shows the different bioresorption behaviors; the b-HA layer on the CCPC increases the resorption produced by osteoclasts.

4. Conclusion

A novel sponge, covered with b-HA on its skeleton surface, was prepared using a particle-leaching technique combined with a biomimetic processing. The formed sponge has numerous, large pores of 450–580 μm in diameter, which are connected with channels having a diameter in the range of 70–120 μm, as well as a high porosity of 75%. The sponge is expected to allow the migration of cells into its interior, and is not broken in normal handling during operations. Cell-compatibility of the CCPC is greatly enhanced after induction of the formation of the b-HA layer. The sponge is one of the great potential candidates as scaffolds for bone tissue engineering.
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