Preparation of Poly(lactic acid) Composite Hollow Spheres Containing Calcium Carbonate, β-Tricalcium Phosphate and Siloxane

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Biodegradable inorganic–organic composite hollow spheres, consisting of poly(L-lactic acid), calcium carbonate, β-tricalcium phosphate and siloxane, were prepared by an oil-in-water emulsion evaporation method to develop an injectable bone substitutes incorporated with cells. The spheres had an almost flat diameter of about 1.0 mm and an open channel of 500–600 μm in diameter on the shell. After soaking the spheres in a simulated body fluid, the silicon and calcium ion-species were gradually released from the spheres. Osteoblast-like cells, marrow stromal cells (MSCs) and osteoblasts migrated into the spheres through the open channel and attached to the inside surface of the sphere shell. The MSCs expressed a high level of alkaline phosphatase on the spheres. Some bone nodules formed on the spheres after the culture of osteoblasts.

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1. Introduction

Organic grafts and synthetics, such as Bioglass® and sintered hydroxyapatite, have been employed in the repair of bone defects.1–5 Recently, much attention has been paid to a new technique called tissue engineering as an alternative approach in the repair of bone defects. Engineering living tissue for reconstructive surgery requires an appropriate cell source, optimal culture conditions and a biodegradable scaffolds as the basic elements.6 The injectable bone substitutes using a syringe play an important role in decrease of risk, polymers are adopted as a matrix phase for preparation of microspheres combined with the slurry containing the emulsions. The hybrids have a high carbonate-containing hydroxy-
apatic-forming ability in simulated body fluid (SBF). The rapid formation of carbonate-containing hydroxyapatite may be attributed to a large amount of calcium carbonate consisting predominantly of vaterite, which has the ability to effectively enhance the supersaturation of carbonate-containing hydroxyapatite because of the fast dissolution of vaterite. In the present work, a hollow in the sphere with a silicon-releasing ability was prepared by w/o/w double emulsions combined with generation of CO₂ gas in the spheres through decomposition of calcium carbonate using composites consisting of PLLA, vaterite, β-TCP and siloxane.

2. Materials and methods

2.1 Preparation of PLLA spheres containing calcium carbonate, β-TCP and siloxane

A total of 0.15 g of a carbodiimide solution was put into 0.33 g of APTES and then stirred for 1 h. It has been reported that carboxy groups are activated by treating a carbodiimide solution. A total of 0.15 g of 0.15 mol/L ammonium solution was added to the APTES and stirred for 1 h to hydrolyze it. An ammonium solution acts as an alkaline catalyst for preventing calcium carbonates and β-TCP, which are subsequently added to APTES, from fast dissolution in an acid region. The hydrolyzed APTES solution was added to 0.5 g of SBF consisting of 10 cm³ of methylene chloride. The mass ratio of PLLA : APTES was 1 : 0.67. Calcium carbonate powders consisting predominantly of vaterite were prepared by a carbonation process in methanol, which has been described in detail elsewhere. The calcium carbonate powders and subsequently pulverized β-TCP powders were mixed with the PLLA solution containing APTES and then the mixture was stirred. The mass ratio of PLLA : calcium carbonate : β-TCP : was 1 : 0.5 : 0.25. Figure 1 shows scanning electron micrographs (SEM photos) of the calcium carbonate and β-TCP. Calcium carbonate particles have the spherical shapes of 0.5–1 μm in diameter. On the other hand, β-TCP particles have squarish shapes of 1–3 μm in diameter.

The sphere was produced by oil-in-water emulsion evaporation. The resultant slurry was emulsified in 30 cm³ of an aqueous solution containing 1 mass% poly(ethylene glycol) as surfactant with constant stirring in an ambient environment for 8 h to evaporate methylene chloride. The spheres formed were collected by vacuum filtration, washed with distilled water and dried in an oven at 40°C at ambient pressure.

2.2 Characterization of the spheres

The crystalline phase of the spheres was identified by X-ray diffraction (XRD) analysis. The morphology of the spheres was observed with an optical microscope and SEM incorporating an energy dispersive spectrometer (EDS). Ion concentrations in 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 after soaking 30 particles of the spheres was analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) to measure the amount of ions released from the sphere.

2.3 Cell cultures

The spheres were sterilized in a steamed autoclave at 120°C for 20 min. The culture was incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. In the present work, three kinds of cells were used in the culture. Osteoblast-like (MC3T3-E1) cells derived from newborn mouse calvaria were used to investigate the migration of cells into the spheres. MC3T3-E1 cells were seeded at a density of 6.0 × 10⁴ cells/well onto about 50 particles of the spheres in a 24-well tissue culture plate. The culture was supplemented with α-modified minimum essential medium containing 10% fetal bovine serum (FBS). Osteoblasts (NFOST OSTEOLAST) received from Cambrex (USA) were used to investigate the formation of bone nodule directly on the spheres. Osteoblasts were seeded at a density of 2.0 × 10⁴ cells/well onto about 50 particles of the spheres in a 24-well tissue culture plate. The culture was supplemented with Dulbecco’s modified Eagles medium (DMEM) containing 10% FBS with 100 mM dexamethasone, 10 mM β-glycerophosphate, 0.05 mM L-ascorbic acid-2-phosphate and 2% penicillin/streptomycin. After these cultures, the samples were fixed in 2.5% glutaraldehyde for 40 min at 4°C and then dehydrated through a series of increasing concentrations of ethanol, and finally dried with hexamethyldisilazane. The morphology of cultured cells on the surface of the sphere was observed with SEM. The differentiation-ability of cultured cells on the sphere was examined to visualize true positive staining using narrow stromal cells (MSCs). MSCs (Cryo hMSC) received from Cambrex (USA) were seeded at a density of 1.0 × 10⁴ cells/well onto about 50 particles of the spheres in a 24-well tissue culture plate. The culture was supplemented with DMEM containing 10% FBS with 100 mM dexamethasone, 10 mM β-glycerophosphate, 0.05 mM L-ascorbic acid-2-phosphate and 2% penicillin/streptomycin. After the culture, the samples were stained with alkaline phosphatase reagents prepared just prior to use (Sigma-Aldrich) and observed with an optical microscope. After culturing the MSCs on the spheres for 7 d, cell extracts were prepared and examined for alkaline phosphatase activity using naphthol staining method, with red color indicating the presence of alkaline phosphatase.

Calcium carbonate/PLLA composite hollow spheres without APTES and β-TCP were used as a control material to compare its alkaline phosphatase activity with that of the spheres prepared in this work. The spheres consisting of calcium carbonate and PLLA are denoted by CCPC spheres, hereafter. CCPC spheres were prepared by oil-in-water emulsion evaporation. The hollow in CCPC sphere was supposed to be formed by numerous CO₂ gas generated by decomposition of calcium carbonate. For the purpose of cell migration into CCPC spheres, an open channel on the surface was produced by a chemical etching method. CCPC spheres were soaked for 3 min in 50% methylene chloride solution diluted with acetone. An open channel ~800 μm in diameter was easily formed on the surface. In our preliminary experiments, the open channel was sufficiently large to allow cell migration into CCPC spheres.

3. Results and discussion

Figure 2 shows a photograph and an XRD pattern of the
spheres. The diameters of spheres were estimated to be ~1 mm. They had an open channel of 500–600 μm in diameter in the shell. The XRD pattern showed that the sphere consists of crystalline PLLA, β-TCP and three kinds of calcium carbonate, i.e., vaterite, aragonite and calcite. The pH of the PLLA slurry containing calcium carbonate, β-TCP and APTES was measured to be ~9. This indicates that calcium carbonate and β-TCP powders in the slurry are unlikely to readily dissolve during the process. The hydrolyzed APTES was drop-wise added to methylene chloride as an organic solvent during the process. As a result, w/o emulsions were formed in the slurry. The spheres formed spontaneously when the slurry was added drop-wise to the solution containing PEG. The spheres are considered to contain emulsions originated from water for hydrolyzation of APTES. Thus, w/o/w double emulsions formed. On the other hand, a trace amount of calcium carbonate may dissolve to generate CO₂ gas in the sphere. After 3 h of the preparation time of the spheres, the spheres showed ease of deformation even when held by tweezers. This indicated that a large amount of methylene chloride, which dissolves PLA, remained in the spheres. Thus, the spheres are not completely solidified after 3 h of the preparation time. At the first stage of the preparation time of the spheres, the PLLA slurry in the spheres is supposed to move toward the surface of the spheres because of centrifugal force generated during stirring, while the emulsions and CO₂ gas in the spheres are supposed to diffuse toward the opposite side. As a result, the emulsions and CO₂ gas aggregate. It is suggested that an open channel in the sphere shell of the sphere is formed by collapsing partial the sphere surface due to leakage of the emulsions and CO₂ gas from the spheres.

![Fig. 2. Morphology and XRD pattern of the spheres.](image)

Figure 2. Morphology and XRD pattern of the spheres. (○) PLLA, (●) vaterite, (□) aragonite, (▲) calcite and (▽) β-TCP.

Figure 3 shows SEM photographs of the spheres. Figure 3 (a) shows the whole image of the sphere. The thickness of the shells in the vicinity of the channel was ~200 μm. Figure 3 (b) shows the inside surface of the sphere shell. Some pores of ~0.5 μm in diameter were observed with calcium carbonate and β-TCP particles at the surface. Figure 3 (c) shows the fracture surface of the sphere shell. Large amounts of calcium carbonate and β-TCP powders were observed in the shell. Note that almost no pores are observed in the shell as shown in Fig. 3 (c). Figure 3 (d) shows the exterior surface of the sphere shell. From the view points of the morphology, numerous calcium carbonate powders were observed on the exterior surface. EDS analysis showed that the sphere includes Si with Ca and P (Si/Ca = 0.05 in atomic ratio). The nominal amount of silicon/calcium element in the prepared slurry was 0.4. More than 100 ppm of silicon species was detected in the solution containing PEG after preparation of the spheres by ICP-AES. A large amount of APTES is believed to be released from the sphere during the preparation process. As a result, a trace amount of silicon species were incorporated in the sphere.

![Fig. 3. SEM photographs of the spheres.](image)

Figure 3. SEM photographs of the spheres. (a) The whole image of the sphere, (b) the inside surface of the sphere shell, (c) the fracture face of the sphere shell and (d) the exterior surface of the sphere shell.
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Fig. 4. Ion concentrations in SBF before and after soaking of the spheres. Soaking time "0" means "before soaking." (•) Ca²⁺, (□) P⁵⁺ and (▼) Si⁴⁺.

These indicate that Ca²⁺ ion is released from predominantly calcium carbonate, not β-TCP, at the initial stage after soaking. β-TCP is considered to remain longer than calcium carbonate. β-TCP combined with PLLA is expected to show a buffering effect for acidic degradation products released from PLLA. The Si⁴⁺ ion species in SBF increased gradually after soaking. Release of Si⁴⁺ ion species is expected to stimulate bone-forming ability on the sphere.

Figure 5 shows SEM photographs of MC3T3–E1 cells on the inside surface of the shell after 5 d of incubation. Numerous cells attached to the inside surface. The diameter of the open channel (500–600 μm) is sufficiently large to allow cells to migrate into the spheres. MSCs were used to evaluate the influence of β-TCP and siloxane in the spheres on the cell differentiation. Figure 6 shows photographs of alkaline phosphatase staining for MSCs on the spheres for 7 d. The cells on the spheres prepared in the present work expressed a higher level of alkaline phosphatase than those on the CCPC spheres. The existence of β-TCP and the released silicon species from the spheres are believed to contribute to cell-differentiation ability on the material. Hench et al. suggested that silicon and calcium ions induce IGF–II to stimulate the formation of bone.

The sphere prepared in the present work showed that silicon and calcium ion-species-releasing ability in a solution. We hypothesized that the sphere containing β-TCP and siloxane would stimulate osteoblasts to form bone nodules in response to dissolution products from the material. Figure 7 shows the SEM photographs of osteoblasts cultured for 14 d on the sphere. Several aggregation formed newly by osteoblasts were observed on the sphere. An EDS result collected from the aggregation showed that the aggregation includes Na and Mg with Ca and P. From the viewpoint of the morphology and the EDS result, these aggregations are believed to be bone nodules. In our preliminary experiments, almost no bone nodules formed on the CCPC spheres after 14 d of incubation using osteoblasts. This may imply that the spheres containing β-TCP and siloxane stimulate mineralization on the substrates.
Fig. 7. SEM photographs of (a) the whole image of the sphere and
(b) the inside surface of the sphere after incubation of osteoblasts for 14 d. (→) bone nodule.

4. Conclusion

PLLA composite hollow spheres containing calcium carbonate, $\beta$-TCP and siloxane were prepared by oil-in-water emulsion evaporation. The spheres of $\sim 1.0$ mm in diameter had an open channel of 500–600 $\mu$m in diameter on the surface. The spheres showed that silicon and calcium ion-species-releasing ability in a solution. In vitro cell culture investigation showed that the open channel is sufficiently large to allow cell migration into the spheres. The existence of $\beta$-TCP and the silicon species released from the material may contribute to promote differentiation of MSCs to osteoblasts and stimulate formation of bone nodules by osteoblasts. The PLLA-calcium carbonate-$\beta$-TCP composite hollow spheres with silicon-releasing ability are promising injectable bone substitutes which cells can be incorporated.

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