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Preparation of hydroxyapatite spheres with an internal cavity as a scaffold for hard tissue regeneration

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Abstract Microparticulates are currently regarded as a useful matrix for the delivery of bioactive molecules and tissue cells. Herein, hydroxyapatite (HA) spherical microparticulates with an internal cavity were produced using an oil-in-water emulsion technique. The HA slurry in the organic solvent was formulated into spherical particles in a water bath containing a surfactant. Rapid evaporation of the solvent helped create a cavity within the microparticulates. The microparticulates were heat-treated at 1,200°C to produce bioactive HA particles with a mean size of approximately 363 μm. Osteoblastic cells were observed to spread and grow favorably over the surface as well as within the cavity of the microparticulates. The currently developed HA microparticulates with an internal cavity are considered to be useful as a scaffolding matrix for bone tissue engineering and direct filling skeletal defects.

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1 Introduction

Microparticulates have been investigated for their potential use in the biomedical fields, such as the delivery of drugs, proteins and cells [1]. A range of polymers, inorganics, and their composites were developed in the microspherical form for this purpose and showed in vitro feasibility and/or clinical performance [2–10]. Many in vitro and in vivo studies reported that microparticulates are effective in retaining and releasing bioactive macromolecules to elicit therapeutic effects at the gene or cellular or tissue level.

Hydroxyapatite (HA) is one of the most-widely used biomaterials in hard tissue repair and regeneration [11]. Small-sized (tens to hundreds of micrometers) HA granules have been synthesized to fill and augment defects in the periodontal pockets and alveolar bones [12–15]. Because of its chemical composition, which is similar to that of inorganic part of natural bone, an excellent bone forming ability has been reported in many experimental models. Moreover, HA has been developed as a scaffolding material for the recruitment of bone-associated cells in bone tissue engineering applications [16–18]. In order to induce the specific biological reactions, including in vivo therapeutic effects and ex vivo cell differentiation for tissue engineering, appropriate bioactive signals, such as proteins, antibiotics and growth factors have been introduced in combination with HA granules and scaffolds [19–22, 25].

Compared with the porous block, the granular type of HA, including the microspherical form, can be used as an injectable carrier as well as a filler for complex-shaped defects with a tunable size and composition [3, 4]. The microparticulates are considered to be a useful vector for the recruitment of osteogenic/stem cells when implanted directly within defective tissues or even for the delivery of certain types of cells after being engineered ex vivo [3–7]. Recent studies on the culturing and delivery of cells using polymeric microparticulates have shown the efficacy of cellular growth and population on the spherical substrate [6, 7]. Moreover, our previous work on collagen-apatite microspheres has shown the potential of microsphere in supporting the growth of mouse-derived stem cells and their expression of bone-associated genes [4]. However, there has been limited studies on the HA microsphere, particularly on its usefulness as a cell delivery and tissue engineering matrix, whilst most focused on the drug delivery of the bioceramic particles or on the control over processing variables involved in the sphere formation [10, 23, 24].

In this study, HA microspheres were produced using a simple water-in-oil emulsion method. In particular, the microspheres were evacuated to create an internal cavity, which is considered to be particularly useful for the delivery of cells as a tissue engineering scaffold. This paper describes the processing route for the generation of HA microsphere with an internal cavity and the assessment of its initial osteoblastic responses.

2 Materials and methods

2.1 Production of HA microspheres

The HA powder was produced from a wet-reaction between the precursors of $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ in a water-based solution. The two chemicals were dissolved each in distilled water and mixed at a stoichiomertic Ca/P molar ratio of 1.67. The reaction proceeded at 37°C for 24 h in air, and the pH was adjusted to 9.0 using a Tris–HCl buffer. After the reaction, the precipitate was filtered, washed, and dried. The dried powders were calcined at 700°C for 3 h to produce the HA powder.

The HA powder obtained was dispersed at 20 wt% in dichloromethane (DCM) containing 5 wt% poly(vinyl buryral) (PVB). After vigorous stirring, the mixture was dropped into a water bath containing 2 wt% poly(vinyl alcohol) (PVA) as a surfactant at 25°C with constant stirring at 350 rpm . The ratio of mixture slurry to water bath was set to 1/30. While stirring, the solvent within the slurry was evaporated, and the microspherical particles were hardened. After stirring for 30 min, the microspheres were gathered and filtered though a filter paper and washed with distilled water. The filtered microspheres were dried overnight at 50°C, and then heat treated in a furnace under the following thermal conditions: heating at 2.5°C/min to 600°C, holding for 4 h, heating at 10°C/min to 1,200°C, holding for 2 h, and air-cooling to room temperature. Figure 1 shows a schematic diagram of the experimental procedures to produce the HA microspheres.

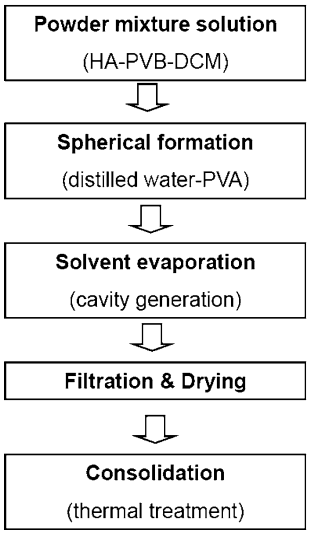

Fig. 1 Schematic shown the experimental steps for preparing the HA microspheres

The crystalline phase of the obtained powders and microspheres was analyzed by X-ray diffraction (XRD). The morphology and microstructure of the microspheres was examined by scanning electron microscopy (SEM). The size distribution of the microspheres was analyzed from the SEM image.

2.2 Cell culturing on the microspheres

Murine-derived preosteoblast (MC3T3-E1) was used to assess the cellular responses to the HA microspheres. Cells were maintained on a culture flask containing a growth medium (α-MEM, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin), supplemented with 10% fetal bovine serum (FBS). For the cell test, the HA microspheres were selected with sizes in the range of 200–600 μm by sieving, and sterilized with 70% ethanol for 30 min and dried under a laminar flow. The microspheres were washed twice with a serum-free medium, and 10 mg of the sample was placed into the individual wells of a 96-well plate. The concentration of the microspheres was chosen to almost fully cover the well surface. As a control, a blank well plate (free of microspheres) was also used. A 50 μl aliquot of the cell suspension (density $= 4 \times 10^5$ cells/ml) was seeded onto each well. The medium was supplemented with 50 μg/ml

ascorbic acid, 10 mM β-glycerol phosphate and 10 nM dexamethasone in order to induce osteoblastic differentiation. After 6 h incubation, a 150 µl aliquot of the medium was added to each well, and then cultured for 3, 7 and 10 days. The cell growth kinetics was measured using an MTS assay. Five replicate samples were tested for each condition ($n = 5$). The cell morphology on the microspheres was observed by SEM after fixing the samples with glutaraldehyde, dehydrating them with a graded series of ethanol, and treating with hexamethyl disilazane. SEM was operated at an accelerating voltage of 15 kV after coating the sample surface with Pt.

3 Results and discussion

3.1 Production and characteristics of HA microspheres

As illustrated in Fig. 1, the HA-PVB-DCM mixture slurry was formulated into spheres via an oil-in-water emulsion technique, wherein PVA was used as a surfactant to mediate the distilled water and the PVB-bound HA. PVB, used as a binder for the HA powder, maintains the initial spherical form of the ceramic powder during heat-treatment. Moreover, DCM, which was selected as a solvent for the HA-PVB mixture, acts as a cavity generator within the microspheres.

Figure 2 shows the XRD patterns of the initially obtained HA powder and the microsphere-formulated sample. The HA powder used as a precursor for the microsphere was initially poorly crystallized (Fig. 2a). When the HA powder was formed into a microsphere and then followed by a heat treatment at 1,200°C, characteristic peaks of a crystalline HA were clearly observed (Fig. 2b).

Figure 3 shows an optical image of the as-prepared HA microspheres before the heat treatment. Spherical-shaped particles with a large cavity (arrowed) were produced well. The cavity was deemed to be created by the rapid evaporation of the DCM solvent during the formulation of microspheres within a water bath. In practice, DCM was observed to evaporate rapidly resulting in the solidification of the remaining HA-PVB within an hour. The resultant HA-PVB particles following the subsequent filtering step were shown to be rigid and hard enough for manipulation.

The as-prepared HA-PVB microspheres were then heat-treated at 1,200°C for 2 h. The amount of weight loss during the heat treatment was measured to be approximately 17% of the initial weight, which was attributed mainly to the elimination of the PVB binder. Moreover, the size of the microparticles was reduced significantly due to the partial sintering of the HA powder.

The SEM morphology of the HA microspheres heat-treated at 1,200°C is shown in Fig. 4. The spherical form

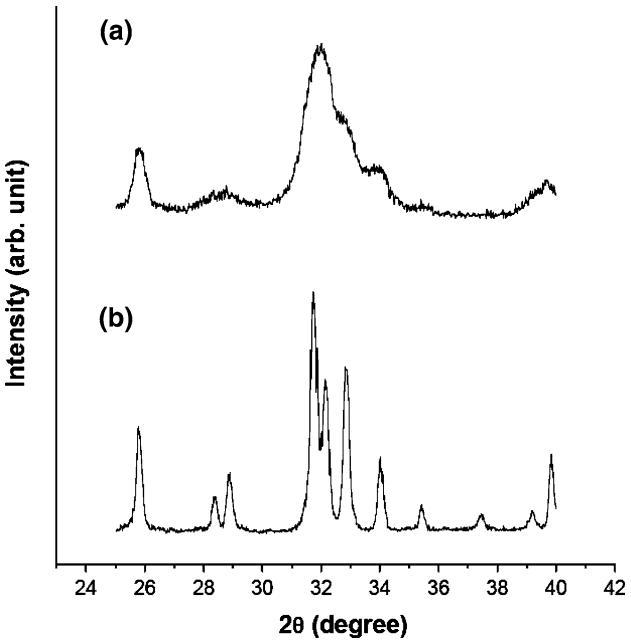

Fig. 2 XRD pattern of the initial HA powder and the HA microsphere produced using the HA powder

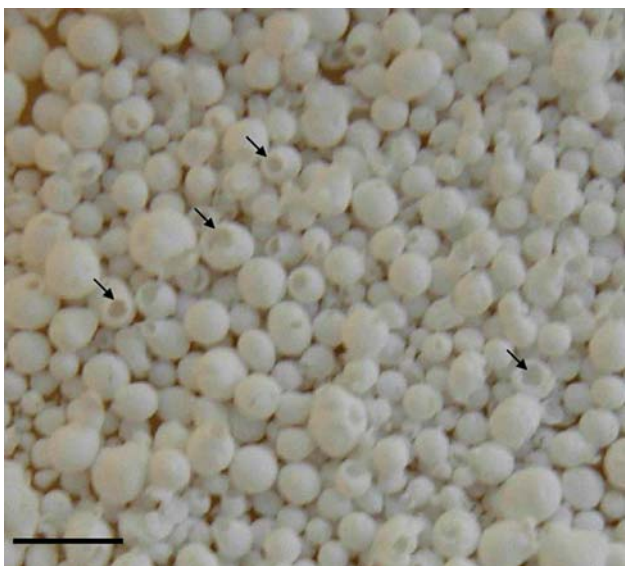

Fig. 3 Optical view of the as-prepared HA microspheres before heat treatment. Note the cavity within each microsphere (arrowed). Scale bar = 1 mm

was maintained after the heat treatment. An opening hole more than ~100 µm in size was clearly observed at one end of the microsphere (Fig. 4a). As a result, the microsphere showed a thin-walled hollow structure (Fig. 4b). The wall thickness was approximately a few tens of micrometers. A higher magnification of the microsphere surface showed a micro-roughened morphology (Fig. 4c), revealing many HA grains (~less than a few micrometers in size). Based on the SEM image, it was concluded that

the HA microspheres feature a thin-walled hollow structure with one end open and rough outer surface. Such a morphological trait is considered to be favorable in the recruitment of tissue cells for the medical applications.

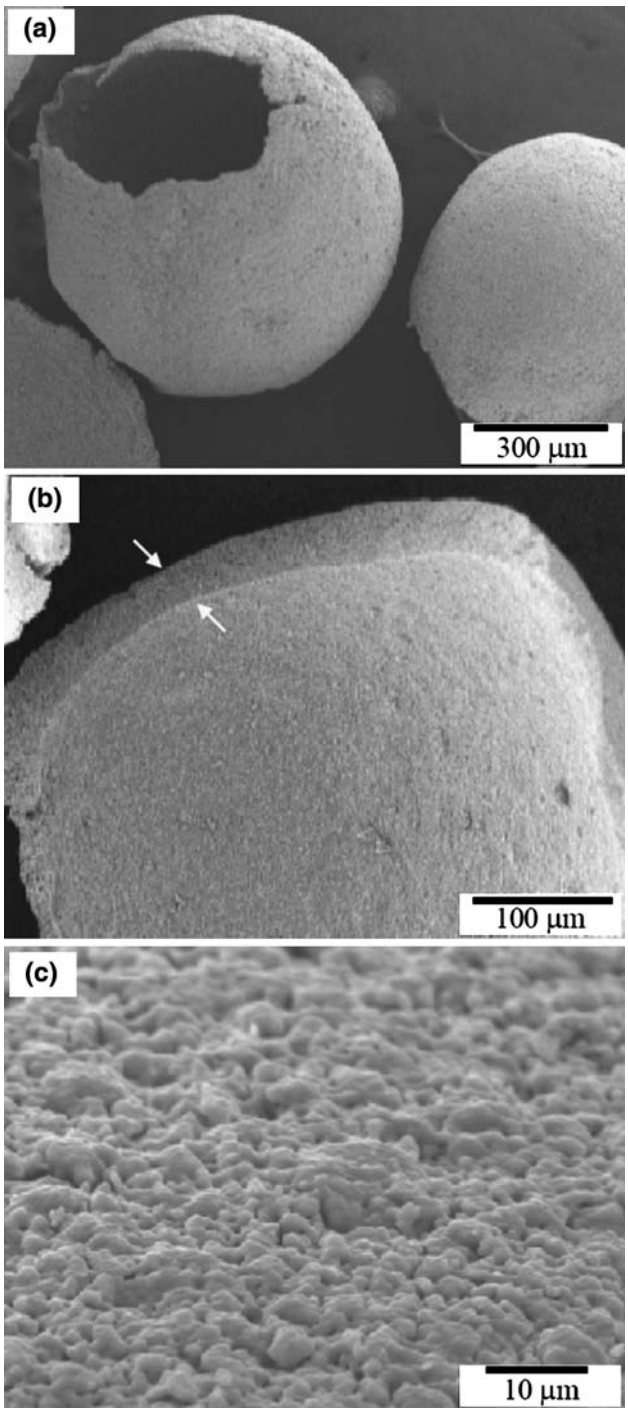

Fig. 4 SEM morphology of the HA microspheres after heat treatment at 1,200°C. (**a**) Image showing an opening hole at one end and (**b**) a thin wall (arrowed) comprising the microsphere. (**c**) High magnification image of the surface showing a micro-roughened granular morphology

Figure 5 shows the size distribution of the HA microspheres. The mean diameter of the microspheres was 363 μm. This size of the HA microspheres is considered to be appropriate for the cell delivery and tissue engineering applications.

3.2 Osteoblast cell growth on the HA microspheres

The feasibility of the HA microspheres in the population and growth of tissue cells for the treatment of skeletal defects was briefly assessed by seeding osteoblastic cells on the microspheres and monitoring the cell growth behavior. Figure 6 shows SEM micrographs of the cells attached and grown on the HA microspheres. The cells were shown to have active cytoskeletal extensions in intimate contact with the underlying micro-roughened substrate (Fig. 6a). Moreover, some cells were viable within the internal surface of the microspheres (Fig. 6b), suggesting the cavity played a favorable role in hosting and populating cells, and the possible applications of the microspherical particles as a scaffold in cell delivery and tissue engineering.

The cell growth level on the HA microspheres was assessed using an MTS method, as shown in Fig. 7. The cells showed an ongoing increase with culturing time, reflecting good cell viability upon the spherical substrate. Based on this, it is considered that the developed HA microsphere, with thin-walled hollow structure and micro-roughened surface, should have potential applications in bone regeneration field. The evacuated inner wall is believed to efficiently recruit and contain cells, and the rough surface morphology is favorable for directing the

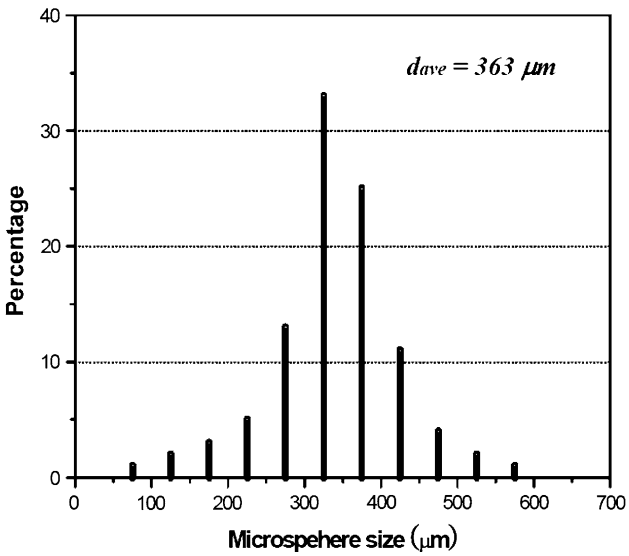

Fig. 5 Size distribution of the HA microspheres after heat treatment at 1,200°C, showing an average diameter of 363 μm

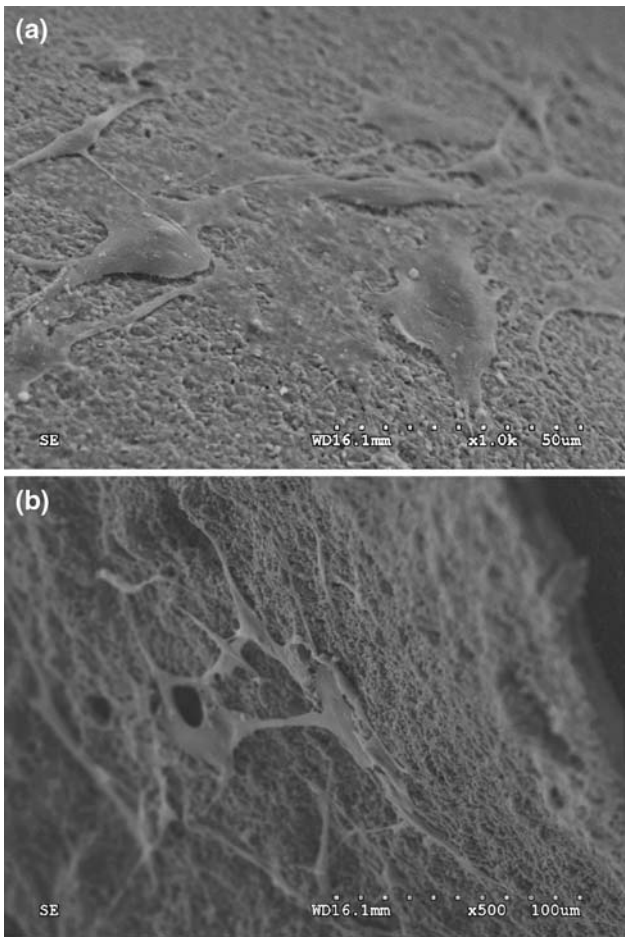

Fig. 6 Electron micrographs of osteoblastic cells grown on the HA microspheres with culturing for 7 days. Cells grew actively with many cytoskeletal extensions on the microsphere outer surface (**a**) as well as on the evacuated inner wall surface (**b**)

initial cell adhesion and subsequent cellular behavior. However, further studies such as three-dimensional culturing of cells on the HA microspheres and the assessments on osteogenic properties will be needed.

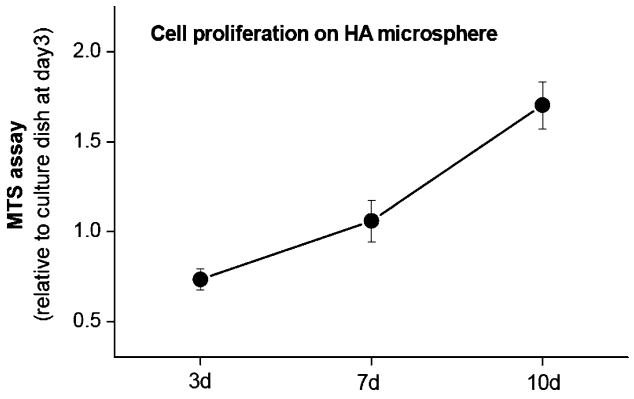

Fig. 7 Cell growth kinetics on the HA microspheres during culturing up to 10 days, showing an ongoing increase with culturing time

4 Conclusions

Microspherical HA particles with an internal cavity were produced for the applications to skeletal defect treatment. A mixture solution of HA powder and PVB binder in dichloromethane was formulated into spherical microparticles within the PVA-containing water bath, and the microparticles were consolidated at 1,200°C. The sintered microspheres showed a thin-walled hollow structure as a result of the rapid evaporation of the solvent. The osteoblastic cells were shown to spread and grow actively on the outer surface as well as on the inner cavity wall of the microspheres. The currently produced HA microsphere with a thin-walled hollow structure is considered to be potentially useful as a scaffold for cell delivery and tissue engineering.

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