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# Uniform tricalcium phosphate beads with an open porous structure for tissue engineering

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ABSTRACT

Uniform tricalcium phosphate (TCP) porous beads with micro and macro pore sizes were fabricated using a simple fluidic device. For micro-porous TCP beads, an aqueous gelatin mixture containing TCP powder was introduced as the discontinuous phase into the fluidic device, where a toluene phase served as the continuous phase. The resulting aqueous TCP droplets were instantly frozen at −20 °C and freeze-dried, followed by calcination at 1200 °C. An oil-in-water-in-oil (O/W/O) emulsion templating method was employed to fabricate macro-porous TCP beads. An oil-in-water (O/W) emulsion was introduced into the fluidic device as the discontinuous phase with all other experimental conditions the same as for the micro-porous TCP beads. Uniform macro-porous TCP beads with a highly porous structure were finally obtained after freeze-drying and calcination. Large pore size and good interconnectivity of the macro-porous TCP beads were confirmed by scanning electron microscopy and porosimetry. In addition, penetration of host tissue into the macro-pores of the TCP beads was demonstrated by subcutaneously implanting the two types of porous TCP beads into mice and histologically analyzing stained sections at 1–4 weeks post implantation. The macro-porous TCP beads with a highly open porous structure could potentially be used as an injectable material for bone tissue engineering.



## 1. Introduction

Tissue engineering is an interdisciplinary field that combines biology, medical science, and engineering to improve quality of life by repairing the function of diseased or damaged tissues. Among the many issues in tissue engineering, a porous construct act as a platform capable of properly guiding the behaviors of cell and tissue, including proliferation, differentiation, penetration, and neovascularization [1,2]. Besides large three-dimensional porous constructs (scaffolds), porous beads with less than 1 mm in diameter have been the focus of much attention due to their injectability into the human body without surgical incision. Many groups have reported on the fabrication of highly porous beads from a variety of natural and synthetic polymers [3–5]. The fabrication methods were mainly based on gas foaming [6,7], melt-molding and particle-leaching [8], air incorporation [9], freeze-drying [10], and emulsion templating [11]. In addition, the superiority of porous beads for viability, proliferation, and differentiation of cells has been confirmed in comparison to non-porous beads [12]. The better performance of porous beads can be attributed to the presence of large and open pores that provide a large surface area for cells and facilitate diffusion of nutrients and oxygen, and penetration of host tissue and blood vessels.

Despite many previous studies on the fabrication of porous beads, most studies have been limited to organic materials of natural (e.g., chitosan, alginate, and gelatin) and synthetic (e.g., poly(ε-caprolactone) (PCL) and poly(D,L-lactide-*co*-glycolide)) polymers. Several groups have prepared porous beads from inorganic materials such as calcium phosphate and hydroxyapatite [13–15]. Descamps et al. had fabricated scaffolds with controllable pore sizes from 100 to 900 μm using a templating method [15]. However, most of the porous beads had few pores with an small size (approximately 1 μm) and were not large enough for cell penetration.

Among many tissues and organs in the human body, there have been a lot of reports on bone substitutes and regeneration [16–19].

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For several decades, bone grafts from inorganic materials (e.g., hydroxyapatite and calcium phosphate) and metals (e.g., titanium and aluminum) have been needed in orthopedic surgery to treat bone defects [20–22]. One of the many materials widely used for bone defects is tricalcium phosphate (TCP) due to approval by the Food and Drug Administration for clinical applications [23,24] as well as its excellent biocompatibility, biodegradability, and bone regeneration properties [25–27].

Here, we demonstrated the fabrication of uniform porous TCP beads with a large and open porous structure using a simple fluidic device based on emulsion templating. For comparison, uniform TCP beads with small pores (approximately 1 μm in diameter) were prepared using the same fluidic device, except for emulsion templating. Penetration of host tissue into the large pores was also demonstrated by subcutaneous implantation of the TCP beads.

## 2. Materials and methods

### 2.1. Preparation of porous TCP beads

Uniform porous TCP beads were produced using a simple fluidic device [28]. The fluidic device with two-way flow consisted of a Tygon® tube (1/32 in. i.d. × 3/32 in. o.d.), a glass microcapillary (0.5 mm i.d. × 1 mm o.d.), and a 26 G needle. Tricalcium phosphate (TCP, Alfa Aesar, Ward Hill, MA, USA), gelatin (type A, from porcine skin, Sigma–Aldrich, St. Louis, MO, USA), poly(ε-caprolactone) (PCL, $M_w \approx 65{,}000$, Sigma–Aldrich), polyvinyl alcohol (PVA, $M_w \approx 13{,}000$–$23{,}000$, Sigma–Aldrich), and toluene (J. T. Baker, Phillipsburg, PA, USA) were used to fabricate the TCP porous beads. An aqueous mixture (10 mL) containing TCP powder (4 g), gelatin (1 g), and zirconia balls (0.5 g, 1 mm in diameter) was vigorously vortexed for 12 h to disperse the TCP powder. After removing the zirconia beads, an aqueous dispersion containing gelatin and TCP powder was obtained. Flow rates of each phase in the fluidic device were independently adjusted using syringe pumps (NE-1000, New Era Pump Systems Inc., Wantagh, NY, USA). For the micro-porous beads, the resulting aqueous TCP dispersion was introduced as a discontinuous phase into the fluidic device with constant heating at 60 °C using a digital temperature controller (HTC210 K, LK Labkorea Inc., Seoul, Korea), where toluene solution containing Span® 80 (3 wt%) served as the continuous phase. In the case of the macro-porous beads, a primary oil-in-water (O/W) emulsion was prepared by emulsifying a PCL solution (2 g, 3 wt% in toluene) in the aqueous TCP dispersion (6 g) with a homogenizer (Dispenser T 10 basic, IKA Works, Inc., Wilmington, NC, USA) at 20,000 rpm for 1 min. The O/W emulsion was introduced as the discontinuous phase into the fluidic device instead of the aqueous TCP dispersion. In both cases, the resulting emulsion droplets were collected in a precooled toluene phase (−20 °C) and placed in a freezer (−20 °C) for 6 h. Finally, porous TCP beads were obtained by freeze-drying and calcination from 25 to 1200 °C for 3 h and another 3 h at 1200 °C.

### 2.2. Characterization of the porous TCP beads

Optical microscopy (IX71, Olympus, Tokyo, Japan) and scanning electron microscopy (SEM, S-4800, Hitachi, Tokyo, Japan) were used to characterize the micro- and macro- porous TCP beads. The average diameters and standard deviations of the porous TCP beads and inner and surface pores were calculated from SEM images by analyzing at least 100 beads for each sample using ImageJ software (National Institutes of Health, Bethesda, MD, USA). In addition, the pore size distributions of the porous TCP beads were evaluated using mercury intrusion porosimetry (Autopore IV 9500; Micrometrics, Londonderry, NH, USA). For the compressive strength test, sample were packed in a cylinder with a 10 mm in radius and a 5 mm in height at a crosshead speed of 1 mm/min using a screw-driven load frame (OTU-05D, Oriental TM Corp., Seoul, Korea).

### 2.3. Subcutaneous implantation of the porous TCP beads and histological assessment

Twenty-four adult male BALB/c mice (5 weeks old, Orient-Bio, Seoul, Korea) were randomly divided into two groups (I for micro-porous and II for macro-porous beads). The animals were anesthetized with Zoletil 50® (10 mg/kg, ip; Vibac Laboratories, Carros, France) and aseptically prepared for implantation of the porous TCP beads. Two parasagittal incisions were made on the dorsum of each animal and subcutaneous pockets were created lateral to both incisions by separating the subcutaneous facial plane using blunt dissection. Approximately 30 porous beads were inserted into each subcutaneous pocket. Skin incisions were then closed with Dafilon (B. Braun Surgical S.A., Rubi, Spain). Three animals in each group were euthanized with carbon dioxide gas and the tissue containing the TCP beads was explanted at 1–4 weeks post implantation. All surgical procedures were performed in accordance with the guidelines of an approved protocol from the Institutional Animal Care and Use Committee (IACUC) of The Catholic University of Korea (Republic of Korea). After explantation, the samples were fixed in 10% formalin (Sigma–Aldrich) for 24 h, dehydrated, embedded in paraffin wax, and sectioned (12 μm in thickness) using a microtome (RM2235 Leica Biosystems, Wetzlar, Germany). The cross sections were mounted on glass slides and stained with hematoxylin and eosin (H&E) to examine the penetration of host tissues into the pores and/or voids among the TCP beads. The as-prepared slides were imaged using optical microscopy.

## 3. Results and discussion

### 3.1. Preparation and characterization of the porous TCP beads

The procedure for fabricating the porous TCP beads included three major steps: preparation of an aqueous TCP dispersion and/or emulsification; production of uniform TCP droplets using a fluidic device; and freeze-drying and calcination. Fig. 1A and B schematically show the fluidic device for producing the uniform TCP droplets in water-in-oil (W/O) and oil-in-water-in-oil (O/W/O) emulsion systems, finally corresponding to micro- and macro-porous TCP beads, respectively. In a typical demonstration, a ball-mill process using zirconia balls was employed to disperse the TCP powder in a water phase containing gelatin. After removing the zirconia balls, the resulting aqueous TCP dispersion was heated to 60 °C and introduced into the fluidic device. Spherical TCP droplets prepared in the fluidic device were collected in a precooled toluene (−20 °C) for gelation, followed by placing in a refrigerator for further freezing. Gelatin in the water phase was used as a gel-forming material to allow for the formation of spherical water droplets in the toluene phase. The aqueous TCP droplets maintained a solid state as ice during freezing, whereas the toluene (melting point, −93 °C) was in a liquid phase. After removing the excess toluene using filter paper, the frozen TCP droplets were freeze-dried and calcined at 1200 °C [29]. Through the calcination, all organic molecules were removed and the calcined TCP powders finally remained. For macro-porous TCP beads, a homogenized O/W emulsion was introduced into the fluidic device as the discontinuous phase instead of the aqueous TCP dispersion. The macro-porous TCP beads could be obtained with other conditions similar to those used for the micro-porous beads. Fig. 1C–F show the optical microscopy images of the resulting water and O/W TCP droplets dispersed in the toluene oil phase. Both droplets were spherical in shape and uniform in diameter with a coefficient of variation (CV) less than 5%. Unlike the water