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# Multi-channel biphasic calcium phosphate granules as cell carrier capable of supporting osteogenic priming of mesenchymal stem cells

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## HIGHLIGHTS

- Multi-channel BCP granule fabricated by multi-extrusion process successfully served as cell platform for MSCs.
- MSCs attached well on the granule's surface, while the micro-channel design allowed cell infiltration within the granules.
- Expression of osteogenic markers (ALP, OPN, COL-1 & RUNX 2) confirm successful priming of MSCs and lineage commitment.

## GRAPHICAL ABSTRACT


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## ABSTRACT

Advances in bone tissue engineering include versatile and intricate biomaterial scaffolds in combination with stem cells for enhanced bone regeneration. In this study, a unique scaffold with multi-channels designed to allow cell infiltration within its pores was investigated for its capability to serve as a stable platform for adhesion and osteogenic priming of mesenchymal stem cells. The biphasic calcium phosphate multi-channel granule consisted of 60% hydroxyapatite and 40% β-tricalcium phosphate. Successful loading and retention of isolated and expanded rat bone marrow-derived mesenchymal stem cells (rBMSCs) were observed. The cells proliferated within the microchannels starting from the surface then into the channels. The multi-channel granules were also able to support osteogenic priming of rBMSCs in 2D culture without the aid of a growth factor. Alkaline phosphatase, type I collagen, and runt-related transcription factor 2 expressions were detected with high osteopontin marker expression in as early as 7 days, which persisted for 14 days of culture under osteogenic condition. Results confirmed commitment towards osteogenic lineage of rBMSCs that have attached and grown onto the surface of the multi-channel granules and thus have high potential as a cell-scaffold based approach in bone regenerative medicine.



## 1. Introduction

Research in regenerative medicine has been extended to the use of cells as therapeutic tools for bone regeneration with several studies demonstrating the ability of mesenchymal stem cells (MSCs) to enhance bone repair and regeneration [1–3]. This stems from the ability of MSCs to undergo osteogenic or chondrogenic differentiation [4,5] and release corresponding growth factors that enhance bone formation [6].

Utilizing MSCs together with biomaterials or scaffolds is usually achieved by first expanding cells, followed by seeding onto scaffolds and finally culturing seeded cells present on scaffolds under appropriate

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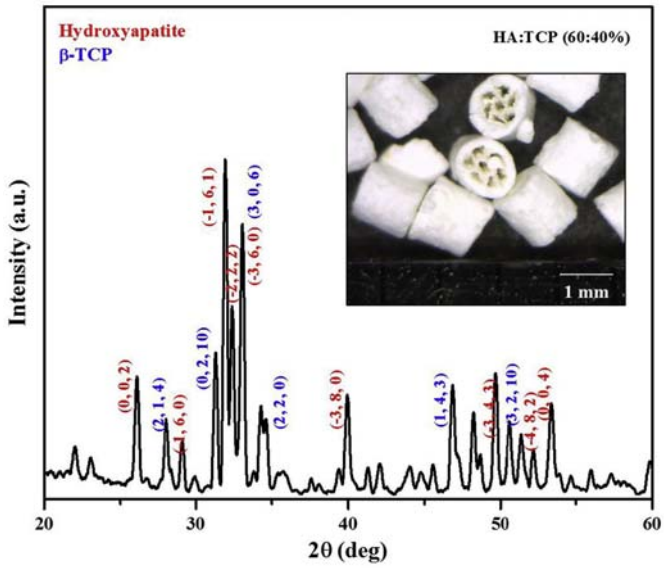


**Fig. 1.** X-ray diffraction spectrum of multi-channel biphasic calcium phosphate granule showing hydroxyapatite and beta-tricalcium phosphate composition with corresponding micrograph of the resulting scaffold (1 mm diameter × 1 mm height, 7 channels).

conditions. The goal is to obtain a homogeneous distribution of viable and metabolically active cells. These key parameters (viable and metabolically active cells) must be satisfied to successfully translate these cells and scaffold based approaches clinically. However, these parameters are affected by material design, composition, geometry, pore size etc. of the scaffold or carrier [7]. In literature, a wide range of biomaterials (natural or synthetic polymers, ceramics and composites) capable of supporting cell attachment, proliferation, differentiation, and ultimately forming new engineered tissues have been proposed [8]. Bioceramic scaffolds have advantageous properties such as porosity, chemical texture, lack of toxic by-products, and ability to promote the differentiation of MSCs and mineralization of formed extracellular matrix [9]. Bioceramic scaffolds have also been previously investigated as potential cell carriers capable of providing stable platforms for cell growth [10]. Yet no cell-scaffold approach has been effectively and fully adapted into the clinical field to date.

In this regard, we designed a cell-scaffold system with high potential for regeneration of bone defects and ultimately clinical translation. The system consists of a biphasic calcium phosphate (BCP) scaffold loaded with a population of osteogenically-primed bone marrow-derived mesenchymal stem cells. BCP-based scaffolds have been known to have bone regenerative properties [11,12]. In particular, BCP constituting 60% hydroxyapatite (HA) and 40% beta-tricalcium phosphate (β-TCP) used herein, which have shown good resorption with osteogenic and bone regenerative properties [13–15]. HA and β-TCP are the most investigated compositions for bone tissue regeneration due to their chemical resemblance to mineral component of natural bone and osteogenic property towards MSCs [16,17]. The BCP scaffold of cylindrical shape with multi-channel design was fabricated via extrusion based technique, which involves the creation of thin filaments passed through a nozzle and layer-by-layer formation or filament combination to build a 3D scaffold [18]. The technique offers control over pore morphology and scaffold architecture as well as reproducibility, and is relatively simple compared with other more recent techniques such as 3D printing [19] and laser sintering [20], which require either a special material, skill or instrument. In vitro validation of the BCP multi-channel granule's capability to serve as a platform and carrier of osteogenically primed MSCs was conducted.

**Table 2**
Specific primer sequences used for real-time PCR.

| Gene | Forward primer (5′ - 3′) | Reverse primer (5′ - 3′) |
|---|---|---|
| GAPDH | AAGTTCAACGGCACAGTCAA | TACTCAGCACCAGCATCACC |
| COL I | CGAGACCCTTCTCACTCCTG | GCATCCTTGGTTAGGGTCAA |
| OPN | GATCGATAGTGCCGAGAAGC | TGAAACTCGTGGCTCTGATG |
| ALP | GACAAGAAGCCCTTCACAGC | ACTGGGCCTGGTAGTTGTTG |

## 2. Materials and methods

### 2.1. Multi-channel biphasic calcium phosphate granules

Multi-channel biphasic calcium phosphate granule (1 mm diameter × 1 mm height) were fabricated by multi-extrusion process as previously described by our group [21,22] with modifications in design and composition. Carbon powder (Sigma-Aldrich, USA) and the ethylene vinyl acetate copolymer (Dupont, Wilmington) were used as pore-former and pore-binder, respectively to shape the composites. The fabrication process involved sequential wrapping of the carbon core with a moulded shell composite of specific diameter to form the filaments. Shell composite (60:40% HA: β-TCP) was prepared by shear-mixing, then casting into a tube-type shell and warm pressing (110 °C) in a cylindrical die. Filaments were further combined and pressed to form the scaffold design. Burnout process was done to remove binders and finally microwave sintering of the scaffold at 1100 °C. X-ray diffraction (XRD, D/MAX-250, Rigaku, Japan) was used to confirm composition of formed multi-channel granules (Fig. 1) with resulting channel diameter ranging from 150 to 300 μm.

### 2.2. Cell isolation, expansion and osteogenic induction

The rat bone marrow-derived mesenchymal stem cells (rBMSCs) were isolated from rat femurs following protocols published elsewhere [23] and kept in growth medium containing α-MEM media (Welgene, Korea) supplemented with 10% fetal bovine serum (FBS, Corning, USA) and 1000 U penicillin/streptomycin (Welgene, Korea). Less than ten passage cells were used in the experiments. Expanded rBMSCs were maintained undifferentiated prior to seeding. Cells were seeded onto 6-well culture plates ($1 \times 10^6$ cells/well) containing 1 g granules. One group was kept in growth medium without osteogenic supplements while the other group was kept in differentiating medium. The differentiating medium consisted of base medium (α-MEM media

**Table 1**
Selected primary antibody used for immunocytochemistry (ICC) and Western blot (WB) analysis with corresponding fluorescent or HRP conjugated secondary antibody.

| Antibody detection (1°) | Source/class | Company | Dilution | Secondary (2°) antibody |
|---|---|---|---|---|
| Alkaline phosphatase (ALP) | Rabbit polyclonal | Santa Cruz Biotechnology | ICC 1:50<br>WB 1:1000 | Anti-Rabbit IgG (H + L), Alexa Fluor® 488 conjugated<br>Anti-Rabbit IgG HRP-conjugated |
| Collagen I (COL1) | Rabbit polyclonal | Santa Cruz Biotechnology | WB 1:1000 | Anti-Rabbit IgG HRP-conjugated |
| Osteopontin (OPN) | Mouse monoclonal | Santa Cruz Biotechnology | ICC 1:50<br>WB 1:500 | Anti-Mouse IgG (H + L), Alexa Fluor® 488 conjugated<br>Anti-Mouse IgG HRP-conjugated |
| Runt- related transcription factor 2 (Runx2) | Rabbit polyclonal | Thermo Fisher Scientific | WB 1:1000 | Anti-Rabbit IgG HRP-conjugated |

F-actin (Red) - Alexa Fluor® 594 (1:100). Nucleus (Blue) – Hoechst 33342.