Microwave sintering and in vitro study of defect-free stable porous multilayered HAp–ZrO₂ artificial bone scaffold

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Abstract
Continuously porous hydroxyapatite (HAp)/t-ZrO₂ composites containing concentric laminated frames and microchanneled bodies were fabricated by an extrusion process. To investigate the mechanical properties of HAp/t-ZrO₂ composites, the porous composites were sintered at different temperatures using a microwave furnace. The microstructure was designed to imitate that of natural bone, particularly small bone, with both cortical and spongy bone sections. Each microchannel was separated by alternating lamina of HAp, HAp–(t-ZrO₂) and t-ZrO₂. HAp and ZrO₂ phases existed on the surface of the microchannel and the core zone to increase the biocompatibility and mechanical properties of the HAp-ZrO₂ artificial bone. The sintering behavior was evaluated and the optimum sintering temperature was found to be 1400 °C, which produced a stable scaffold. The material characteristics, such as the microstructure, crystal structure and compressive strength, were evaluated in detail for different sintering temperatures. A detailed in vitro study was carried out using MTT assay, western blot analysis, gene expression by polymerase chain reaction and laser confocal image analysis of cell proliferation. The results confirmed that HAp-ZrO₂ performs as an artificial bone, showing excellent cell growth, attachment and proliferation behavior using osteoblast-like MG63 cells.

Keywords: bioceramic, hydroxyapatite (HAp), extrusion, cortical bone, osteoblast cell

1. Introduction
Autografting, allografting and artificial grafting have been used to treat and replace bone defects in orthopedic surgery. However, both autografting and allografting methods have limited availability and present pathogen transmission risks from the donor to the recipient. As a consequence, artificial grafting is being increasingly investigated as a means of providing regeneration scaffolds for bone replacement. The aim of grafting is to aid in replacing the missing or damaged tissue and to reinforce the treated area by inspiring new bone ingrowth. This new bone should ideally replace the bone graft through repeated remodeling cycles, enabling repair of the defect site to maintain an optimal balance between form and function. Nowadays, different types of synthetic material such as polymers, ceramics and glasses are being used in osseous defect sites as bone substitutes [1–4]. Calcium phosphate ceramics such as hydroxyapatite (HAp, Ca₁₀(PO₄)₆(OH)₂) or tricalcium phosphate (α-TCP, β-TCP, Ca₃(PO₄)₂) are widely used in clinical practice as bone fillers owing to their favorable chemical composition, excellent biocompatibility and bioactivity [5–7]. Recently, the development of porous HAp and other related calcium phosphates for use in bone treatment applications has become an increasingly important research subject for many medical and materials science fields [8–10]. It has been reported that the use of porous HAp/TCP in vivo promotes bone ingrowth and osteoconduction [11–13]. The chemistry of the bioactive
bioceramic materials that are used to repair the defect region is also important, as it is responsible for supporting the direct bonding of bone to its surface, greatly enhancing its performance over materials that are only bioinert or biocompatible [14]. Recently, studies on using biomimetic modeling to fabricate artificial bone have been carried out. The distinctive characteristics of hard tissues in natural bone are the Haversian lamellae, which comprise columnar osteons, the distribution of osteons into the shape of concentric circles about a central axis, and the connections between the osteons. The morphology of the natural bone structure often provides an optimum compromise between low weight and mechanical strength. Natural bone consists of a porous composite with an interesting structure that exhibits a density gradient as well as anisotropic properties. In particular, pores with a particular dimension and morphology promote bone ingrowth and osteoconduction. Therefore, the porosity gradient, mimicking the bimodal structure of bone (cortical and cancellous), and a sufficient degree of interconnectivity are the most important criteria for developing materials suitable for clinical applications [15]. However, these properties also weaken the scaffold. Therefore, HAp has been reinforced by ZrO2, Al2O3 and other ceramic oxides [16] to make sintered composites. A layered microstructure can be particularly important for improving the mechanical properties; however, it has some inherent problems. During the cooling period of the sintering schedule, thermal expansion mismatch can cause residual stress that can exceed a critical limit and results in cracks, delamination or even catastrophic failure of the material. This can be countered by a gradient layer with intermediate thermal expansion properties. Extrusion processes have been extensively used to make unidirectional porous bodies [17, 18]. However, the technique of making channeled pores and designing a preform with bimodal porosity paved the way for the fabrication of large segments to replace the damaged natural bone. This artificial bone replacement preform is composed of a central hollow to house the bone marrow and an outer shell with channeled porosity, which can be modified into cortical bone after osteointegration. We fabricated a microarchitectural bone repair unit with an aim to replace a section of the long bone using ZrO2 and TCP, thereby combining the strength of ZrO2 and the biocompatibility of TCP. However, the microstructure control was very complicated for this design. The conventional sintering process produced many cracks and the preform was mechanically unstable. Moreover, at the high sintering temperature of 1450 °C for ZrO2, there is an unwanted side reaction that may detrimentally affect the biocompatibility. Therefore, in this work, we employed microwave sintering to make the sintered preform, and optimized the densification of the fabricated body without significantly dispensing the original constituent phases and a mechanically stable preform. We also improved the preform by using optimal sintering conditions and conducting a more detailed investigation into the biocompatibility. These results show potential for fabricating a mechanically stable artificial bone preform imitating natural bone.

In this study, we attempted to fabricate the desired shape of the artificial bone, similar to that of the natural bone, by an extrusion process and by a subsequent binder burnout and microwave sintering. A gradient layer of HAp-HAp-(t-ZrO2)-t-ZrO2 was the key to subduing the effect of thermal expansion mismatch. Microwave sintering was employed to achieve quick densification without significant grain growth, reducing the possibility of chemical reaction among the layers. Compared with a conventional sintering process, the microwave sintering resulted in higher mechanical stability and morphological integrity. Material properties and the morphological aspects were investigated in detail in terms of varying sintering temperature and were analyzed using x-ray diffraction (XRD) and scanning electron microscopy (SEM). The optimum scaffold was further investigated for its biocompatibility in vitro.

2. Experimental procedure

2.1. Extrusion process for fabricating porous composites

2.1.1. Materials. t-ZrO2 was purchased from Tosoh Japan (TZ-3Y, particle size 70 μm) and HAp nanopowders synthesized by an ultrasonic-assisted process were used as the starting powder. Ethylene vinyl acetate copolymer (EVA) was purchased from DuPont USA (ELVAX 210A) and used as the thermoplastic binder for the shaping of composites. Carbon powder (<15 μm, Aldrich USA) was supplied as a pore-forming agent, and stearic acid (Daegung Chemicals and Metals Co. Korea) was used as a lubricant to ensure good mixing of the components.

2.1.2. Microstructure design and extrusion. The fabrication process is described in our previous report [19]. All the powders (HAp, t-ZrO2 and carbon) were shear mixed with EVA and stearic acid separately or in mixture. The first shell composite (45 vol% HAp, 45 vol% polymer and 10 vol% stearic acid) was prepared with a shear mixer (Shina Platec, Korea), and was used to make a tube-type shell (3 mm in thickness) by warm pressing in a cylindrical die at 110 °C. The second shell composite (46 vol% HAp-(t-ZrO2), 43 vol% polymer and 11 vol% stearic acid), the third shell composite (40 vol% t-ZrO2, 50 vol% EVA and 10 vol% stearic acid) and the carbon composite (50 vol% carbon powder, 40 vol% polymer and 10 vol% stearic acid) were prepared using the same process. The HAp-(t-ZrO2) mixture powder in the second shell composite was mixed at the ratio of 75 vol% / 25 vol% by ball milling.

The carbon core (50 vol% carbon, 40 vol% polymer and 10 vol% stearic acid) was extruded in the cylindrical die at 110 °C to make a carbon rod of 22 mm diameter. Two types of carbon core were needed for the experiment; the first type of carbon core was wrapped by the first shell composite (45 vol% HAp, 45 vol% polymer and 10 vol% stearic acid), the second shell composite (46 vol% HAp-(t-ZrO2), 43 vol% polymer and 11 vol% stearic acid) and the third shell composite (40 vol% t-ZrO2, 50 vol% EVA and 10 vol% stearic acid). This was used to make the first-passed filaments 3.5 mm in diameter. The second type of carbon core was needed for the final extrusion to make the central hollow space. It
was wrapped by the first shell composite (45 vol% HAp, 45 vol% polymer and 10 vol% stearic acid) and extruded with a diameter of 10 mm.

The first-passed filaments (diameter 3.5 mm) were fabricated using the extrusion process, which assembled one carbon core and the previously mentioned 3 shell layers fabricated using the extrusion process, which assembled in a cylindrical die. The 52 first-passed filaments were wrapped by the first shell composite (45 vol% HAp, 45 vol% polymer and 10 vol% stearic acid) and assembled in a cylindrical die to obtain the second-passed filaments with a diameter of 3.5 mm. For the final extrusion, a three-layer arrangement with the second type of carbon core were assembled in a cylindrical die with a diameter of 30 mm; the first layer was fabricated using shell composites with 45 vol% HAp, 45 vol% polymer and 10 vol% stearic acid (3 mm in thickness), the second layer consisted of 20 second-passed filaments (3.5 mm in diameter), while the third layer was composed of 15 second-passed filaments (3.5 mm in diameter) and a second carbon core of 10 mm diameter was located in the central axis. The finalized small artificial bone was constructed with a structure similar to that of natural bone through the extrusion and arrangement processes.

2.2.3. RT-PCR reaction (materials and methods). Cells grown in a T75 flask with the extracted solution were collected using Trypsin (EDTA (TE) and washed twice with PBS. Then, the total RNA from the cells was isolated using a Nucleosip RNA II total RNA isolation kit (Germany). cDNA was synthesized using the iScript TM cDNA kit (Bio-Rad, USA) in accordance with the manufacturer’s instructions. Then, 1 µl of the first strand cDNA template was subjected to real-time polymerase chain reaction (RT-PCR) using SYBR Green Supermix (Bio-Rad, USA) with 4 primer pairs (GAPDH, Col, ON, BSP). RT-PCR was performed on a sample that was pre-denatured at 94°C for 3 min, flowed through 40 cycles (denaturation at 94°C for 20 s, annealing at 60°C for 20 s and extension at 72°C for 30 s), followed by a melting analysis between 65 and 94°C.

2.2.4. Cell proliferation. MTT assay. An autoclaved sample was put inside 24 wells of a plate and seeded with 20 000 cells per well. The scaffolds were incubated for 1, 3 and 5 days. After incubation, the samples were transferred to a new plate (24 wells). A 200 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added onto the composite, which was then incubated at 37°C for 3.5 h, and the MTT solution was discarded. Then, the dimethyl sulfoxide (DMSO) solution was added to dissolve any insoluble formazan crystals present on the samples. From the 24-plate well, 150 µl of solution was transferred to a plate with 96 wells. The absorbance was measured at 595 nm using an ELISA reader (Turner Biosystems CE, Promega Corporation, USA) for MTT testing.

Evaluation of protein components of cells grown in extracted solution by SDS-PAGE. To determine the protein component of the cells, 0.1 g of sterile interconnected porous HAp/HAp-(t-ZrO2)/t-ZrO2 was put into 1 ml of DMEM and shaken for 24 h at 37°C; then the solution was used to grow MG-63 cells. About 10^6 cells were seeded on a 6-well culture plate with the extracted solution or with DMEM only (control), and the cells were collected after 1, 3 and 5 days. Protein components of these cells were collected using RIPA lysis buffer (radio-immunoprecipitation assay, Millipore), checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250.

Osteoblast cell adhesion and proliferation. Osteoblast cells (MG-63) were seeded on tissue culture plates as control samples and on the HAp/t-ZrO2 scaffold at a concentration of 10^6 cells cm^-2 in DMEM. For short (30 and 60 min) and long tests (1, 3 and 5 days), samples were kept at...
37°C in a humidified air with 5% CO₂. After incubation, cellular constructs were harvested, rinsed twice with PBS to remove non-adherent cells and subsequently fixed with 2% glutaraldehyde for 15 min and washed with PBS 2 times (15 min per time). The samples were then dehydrated using a series of graded ethanol (EtOH) solutions. Finally, the samples were washed 2 times with hexamethyldisilazane. All the samples were air-dried overnight. Dry cellular constructs were sputtered with gold and observed by SEM.

**Immunofluoresence staining.** Immunohistochemical analysis was performed to assess cells grown on sterile plastic coverslips and the sterile HAp/t-ZrO₂ scaffold. Following treatments, the cells were washed three times in PBS and fixed in 4% paraformaldehyde in PBS for 15 min. After being washed three times in PBS, the cells were permeabilized for 15 min with 0.5% Triton X-100 and for 1 h at room temperature with a blocking buffer containing 5% bovine serum albumin. The cells were then incubated with rabbit polyclonal anti-β-actin (1:1000) overnight at 4°C in a humidified box. After washing three times in PBS, the samples were incubated at room temperature in a humidified box for 1 h with a 1:1000 dilution of Alexa Fluor-conjugated secondary antibody (goat anti-rabbit Alexa Fluor 594) in a blocking solution and again washed with a buffer. The nuclei were stained with 0.234 mg mL⁻¹ 40, 60-diamidino-2-phenylindole (DAPI) for 5 min, and the samples were washed three times in the washing buffer. Coverslips were then mounted on slides using antifade mounting medium (Molecular Probes). Localization of β-actin and cell proliferation were analyzed using an Olympus FV-10 confocal microscope.

**Statistical analysis.** Each experiment was repeated at least 3 times on different days and the data were expressed as the mean ± standard deviation.

3. Results

Figures 1(a), (c) and (e) show optical cross-sectional images of composites sintered by microwaves at different temperatures. Figures 1(b), (d) and (f) are optical images of external surfaces produced under the same three sintering conditions, respectively. Composites sintered at 1300 and 1500°C show a lack of densification or extensive cracking. In contrast, the compacts sintered at 1400°C exhibit minimal cracking and better densification. Figure 2(a) shows a cross-sectional SEM image of the frame region, in which there are three layered composites around the pore: the first layer is HAp, the second layer consists of HAp (75 vol%) and t-ZrO₂ (25 vol%), and the third layer is t-ZrO₂. The second layer was added to prevent the formation of microcracks in the frame owing to the different thermal expansion coefficients of HAp and t-ZrO₂. As demonstrated in figure 2(a), these three layers...
show the values of the external, internal and frame diameters. The interfacial boundaries are intact and do not show any delamination or debonding. The growth of HAp grains is significant at 1500 °C, and extensive cracks and cavities in the microstructure are seen. These results suggest that 1400 °C is the optimal sintering temperature.

Figure 4 shows the phase transformation of HAp/t-ZrO2 composites sintered at (c) 1300, (d) 1400 and (e) 1500 °C. The XRD profiles in (a) and (b) show raw powder peaks of HAp and t-ZrO2. In the samples sintered at 1300 °C, peaks of the HAp and t-ZrO2 precursors were detected without any phase change. However, after sintering at 1400 °C (d), the HAp peaks started to weaken owing to the phase transformation of HAp, and the α-TCP peaks emerged. As the sintering temperature was raised to 1500 °C (e), the HAp peaks disappeared and CaZr4O9 appeared as a reaction product phase. Its peaks were strong compared with the residual t-ZrO2 peaks, confirming that the t-ZrO2 phase was converted mostly to CaZr4O9.

Table 1 shows the values of the external, internal and pore diameters to be approximately 9 mm, 2 mm and 83 μm, respectively. These dimensions were decided arbitrarily, as a close approximation of small bones like those of toes or fingers. The exact dimensions were determined on the basis of the extrusion ratio and the arrangement of the filaments and shell in the die before the last extrusion. Note that it is possible to custom-design the external shape of the fabricated bone in the die with the extrusion process.

Figure 3 shows longitudinal SEM images of HAp/t-ZrO2 composites sintered at 1300 (a), 1400 (b) and 1500 °C (c). The growth of HAp grains is significant at 1500 °C, and extensive cracks and cavities in the microstructure are seen. These results suggest that 1400 °C is the optimal sintering temperature.

Table 1. Material properties of the HAp/t-ZrO2 composites.

| Sintering Temp (°C) | Haversian canal width (μm) | Internal diameter (mm) | External diameter (mm) | Frame thickness (μm) | Compressive strength (MPa) |
|---------------------|---------------------------|------------------------|------------------------|----------------------|---------------------------|
| 1300                | 91 ± 15                   | 2.1 ± 0.5              | 7.3 ± 0.7              | 31 ± 10              | 7.4 ± 1.2                 |
| 1400                | 80 ± 20                   | 2.3 ± 0.4              | 7.6 ± 0.6              | 38 ± 10              | 20 ± 2                    |
| 1500                | 90 ± 12                   | 2.3 ± 0.5              | 8.4 ± 1.0              | 38 ± 7               | 10 ± 2                    |

Figure 4. XRD patterns of (a) HAp powder calcined at 750 °C, (b) t-ZrO2 powder, (c–e) HAp/t-ZrO2 composites sintered at 1300, 1400 and 1500 °C, respectively.

have no cavities or fine microcracks. Figures 2(b), (c) and (d) show the energy-dispersive x-ray spectroscopy (EDS) profiles taken from the regions marked as P, Q and R in the SEM image of figure 2(a). They reveal that the layers were not blended through the extrusion process.
ON and BSP as gene markers expressed in MG63 cell line and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. The RNA of the MG63 cell line grew in an extracted solution of HAp/t-ZrO2 composite; it was isolated and used to synthesize cDNA. Then, the cDNA was used for detecting the gene expression. A positive control was established with MG63 cell line grown in DMEM only. We chose the GAPDH gene as the control for the intact extraction of total RNA, and the expression levels of the Col, ON and BSP genes were tested. As shown in figure 5, the threshold cycle (Ct) values did not vary much between the cells grown in DMEM and those grown in the extracted solution. The expression of GAPDH was the highest (∼15 cycles) and that of BSP was the lowest (34 cycles). Otherwise, ON and Col were expressed after 17 and 21 cycles, respectively. Our results show that the extracted solution, HAp/t-ZrO2 composite, did not result in any toxicity for the expression of marker genes on the MG63 cell line.

To evaluate cell proliferation on the scaffold, MTT and SDS-PAGE methods were employed. Figure 6(a) shows the osteoblast cell proliferation on HAp/t-ZrO2 after 1, 3 and 5 days of culture. The histogram reveals that osteoblast cells grew and proliferated excellently on the HAp/t-ZrO2 scaffold, which is evidenced by the increase in the optical density measured at a wavelength of 595 nm. For example, the optical density was approximately 0.3 after 1 day of culture; however,
shows the attachment, adhesion and growth of osteoblast cells to proliferate. The ability of osteoblast cells to proliferate on the control and HAp/t-ZrO$_2$ composite was determined using SEM. In this experiment, the HAp/t-ZrO$_2$ composite in 10% FBS or in only DMEM with 10% FBS, was used to culture the MG-63 cell line. The cells were collected after 1, 3 and 5 days. Then, all the cell samples were suspended in equal volumes of lysis buffer and loaded to the same volume in SDS-PAGE. The protein content of the cells increased with the culture time. On the first day, the low yield of the cell was in accordance with the small protein concentration. However, the protein concentration rose after 3 and 5 days of culture. The protein components of osteoblast cells grew on normal DMEM similar to the extracted solution. This result matched those of the MTT assay; which suggests that the HAp/t-ZrO$_2$ composite is a suitable material for bone cell adherence and growth.

Figure 7 shows the attachment, adhesion and growth of osteoblast cells on the HAp/t-ZrO$_2$ scaffold for incubation times of 30 and 60 min. Osteoblast cells attached very well on the HAp/t-ZrO$_2$ scaffold within 30 min. Filopodia and lamellipodia appeared and adhered on the HAp/t-ZrO$_2$ surface, as shown in figures 7(a1) and (a2). When the incubation time was increased to 60 min, as shown in figures 7(b1) and (b2), osteoblast cells spread and covered the scaffold surface. Surrounding the cell nucleus, lamellipodia spread and attached onto the scaffold. Comparison between the 30 and 60 min results showed that cells were grown with sizes ranging from approximately 20 to 30 µm.

Figure 8 shows the ability of osteoblast cells to proliferate on the HAp/t-ZrO$_2$ scaffold for 1, 3 and 7 days and compares these abilities with that of the control (tissue culture polystyrene). Figures 8(a1)–(c1) show osteoblast cell proliferation on the control sample, while figures 8(a2)–(c2) show the locations of osteoblast cells on the HAp/t-ZrO$_2$ scaffold. The arrows in figures 8(a2)–(c2) indicate the distribution of osteoblast cells on the scaffold surface. Osteoblast cells attached, spread and proliferated excellently on the scaffold after 1, 3 and 7 days of incubation. A few osteoblast cells adhered on the scaffold surface after incubation for 1 day (figures 8(a1) and (a2)). A comparison of osteoblast cells on scaffolds and on control surfaces reveals that the control surface had more cells. After 3 days of incubation (figures 8(b1) and (b2)), the confluence of osteoblast cells increased and they were distributed around the scaffold hollow (figure 8(b2)). Also, the osteoblast cells showed excellent interaction, as can be seen in figure 8(b2). Osteoblast cells cling to each other especially well after 7 days of culture (figure 8(c)). The density of osteoblast cells was increased and migration of cells toward the hollow was evident. Figure 8(c1) reveals that osteoblast cells on the control sample were aligned. In addition, the surface of the HAp/t-ZrO$_2$ scaffold was covered by osteoblast cells (figure 8(c2)). Osteoblast cells almost closed the pores, and cell filaments almost attached to each other.

To show more clearly that osteoblast cells proliferated excellently on the HAp/t-ZrO$_2$ scaffold, immunofluorescence images of osteoblast cell proliferation on the HAp/t-ZrO$_2$ scaffold surface were taken after 3 (figures 9(a1)–(a3)) and 7 days (figures 9(b1)–(b3)) of culture. Figures 9(a3) and (b3) show the scaffold including the nuclei and β-actin filaments on the scaffold, while figures 9(a2) and (b2) show the nuclei of osteoblast cells stained with DAPI. The actin filaments of osteoblast cells were stained with Alexa to prove that the cells proliferated on the HAp/t-ZrO$_2$ scaffold, which is shown in figures 9(a1) and (b1). These results reveal that osteoblast cells proliferated excellently during 3 and 7 days of culture. The density of osteoblast cells became very high, as shown in figures 9(b2) and (b3) compared with that in figures 9(a2) and (a3) owing to an increase in the number of nucleus cells.
4. Discussion

The final aim of this research was to make an artificial bone preform for small finger-like bones in humans with sufficient mechanical and morphological stability, and we investigated the microwave sintering of a layered composite preform to achieve this goal. The dimensions of the artificial bone preform were designed accordingly [21, 22].

The microwave sintering process yielded a workable artificial preform with high mechanical reliability and good biocompatibility. The important factors that determine the compliance of an implant with the human body are mechanical stability and superior osteocompatibility. The results show that our implant system is suitable for integration with the host bone. The drawbacks of the pure bioactive ceramics like hydroxyapatite and TCP are their mechanical weakness and the difficulty of controlling their porosity to make a suitable microenvironment for bone growth. Several bioceramics such as ZrO$_2$ and Al$_2$O$_3$ have mechanical properties superior to those of calcium phosphate-based ceramics, but are inherently bioinert and pose problems for the final integration of the implant. The use of a composite is an alternative approach. Considering the very fine dimensions of the porous internal frame and the more favorable biocompatibility of a surface comprised of only calcium phosphate, a layered microstructure composite appears to be the best option. However, the thermal expansion mismatch among the layers can cause cracking, interfacial failure and instability of the scaffold. If these problems are solved and the materials can be designed so as to reduce the effect of bioinert surface interaction with the host site, they can strengthen the bioactive calcium phosphate-based ceramics without sacrificing their superior biocompatibility.

In our design, this particular difficulty is addressed using a layered microstructure with a compositional gradient.

Conventional cosintering of HAp and ZrO$_2$ at high temperatures is problematic owing to reactions producing undesirable products that severely limit the biocompatibility. These reactions are promoted by increasing the dwelling time at the sintering temperature, which is otherwise necessary for proper sintering. This problem can be avoided by choosing a sintering method that ensures sufficient sintering within a short time, such as microwave sintering. In this method, the heating rate is relatively high and the dwelling time is significantly shortened, which hinders undesired reactions and, hence, preserves the biocompatibility of the intended materials. In a previous study, we employed conventional sintering, which quickly degraded the HAp phase [19]. However, with microwave sintering at the optimum temperature of 1400°C, the HAp phase was preserved.

The compressive strength of the fabricated porous scaffold still does not match that of natural bone owing to the very important prerequisite of high porosity. We tried to fabricate a bone preform that can be strong enough to maintain a reasonable load during the natural healing period, and at the same time offers extensive porous space for the bone regeneration to take place throughout the whole scaffold. Osteoblast cells are responsible for bone formation. Since the HAp/t-ZrO$_2$ scaffold is designed for use as an artificial bone, it should be tested for osteoblast cell responses such as cell adhesion, cell spreading and cell proliferation. RT-PCR, SDS-PAGE and MTT assay (figures 5 and 6) showed that the scaffolds were suitable supports for cell attachment and proliferation. Optical density data reveal that the density of osteoblast cells increased almost two-fold after 2 days.
of culture, which is similar to the replication cycle of the cells grown [23]. Foreign biomaterials intended for use as artificial bone should interact with bone cells such as osteoblast cells [24]. This interaction should be expressed by cell adhesion, spreading and proliferation. To test the cell attachment, osteoblast MG-63 cells were cultured on the HAp/t-ZrO$_2$ scaffold for 30 or 60 min, and the results are shown in figure 7. The excellent spreading adhesion of the osteoblast cells within 60 min suggests that the HAp/t-ZrO$_2$ can be used as a substrate for cell growth. To confirm that the HAp/t-ZrO$_2$ scaffolds are good substrates for artificial bone applications, longer cell cultures for 1, 3 and 7 days were carried out and assessed by SEM (figure 8) and confocal microscopy (figure 9). Figure 8 shows the SEM image of osteoblast cells proliferated on the control and the HAp/t-ZrO$_2$ scaffold for 1 day (figures 8(a1) and (a2)), 3 days (figures 8(b1) and (b2)) and 7 days (figure 8(c1) and (c2)) of culture. In the case of 1 day culture, osteoblast cell attachment on the scaffold was extensive. In addition, figure 9 shows confocal images of osteoblast cell proliferation on the HAp/t-ZrO$_2$ scaffold after 3 and 7 days culture. The density of osteoblast cells increased with the culture time as shown in figures 8 and 9. Figure 9 shows more clearly the distribution of osteoblast cells through stained nuclei and β-actin filaments. These data confirmed again the distribution and magnification of osteoblast cells on the HAp/HAp(t-ZrO$_2$/t-ZrO$_2$ scaffold toward the hollow. This distribution shows potential for building natural bone along the hollow that occurs at the interface with artificial bone.

The in vitro data revealed the superior biocompatibility of the fabricated bone preform. The structural conformity and phase stability in the microwave sintering method confirm that, by using microwave sintering, it is possible to fabricate an artificial bone scaffold with the required biocompatibility.

5. Conclusions

HAp/HAp(t-ZrO$_2$/t-ZrO$_2$ composites, which were designed as a substitute for small bones such as finger bones, were fabricated by an extrusion process. The samples showed a significant variation of the compressive strength with sintering temperature in the range 1300–1500°C. The highest compressive strength of 20 MPa was achieved at 1400°C without the appearance of any cracks. The Haversian canal and internal diameter are important factors of the microstructure that were respectively optimized to 80 μm and 1.9 mm to promote cell growth. In an in vitro study, the HAp/HAp(t-ZrO$_2$)/t-ZrO$_2$ scaffold demonstrated excellent cell attachment and proliferation as evaluated through cytotoxicity tests, SEM observations and confocal microscopy.

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