Effectiveness of Various Sintering Aids on the Densification and *In Vitro* Properties of Carbonated Hydroxyapatite Porous Scaffolds Produced by Foam Replication Technique

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Abstract

Synthetic carbonated hydroxyapatite (CHA) ceramics are considered as future materials for bone substitutes due to their good bioactivity, biocompatibility and similarity to the inorganic mineralized phase of bone. However, the limited thermal stability of CHA-based materials at elevated temperature remains a critical challenge particularly in producing three-dimensional (3D) porous scaffolds. To address the aforementioned limitation, this paper presents a new approach by incorporating several types of sintering aids, namely $Mg(OH)_2$, $Ca(OH)_2$, NaOH, KOH and K_2CO_3 into the CHA slurry composition to identify the most effective ones in developing 3D CHA scaffolds. This approach focused on physico-chemical, mechanical and biological characteristics that can be helpful in designing scaffolds for bone tissue engineering. Five compositions of scaffolds were prepared by replication technique, sintered at 800°C and eventually cooled down in wet CO_2 atmosphere. Scaffolds prepared with K_2CO_3 (CHAKC) as sintering aid exhibited optimum interconnected pores with densified struts and the highest compressive strength. Biologically, CHAKC provides the most favorable milieu in supporting apatite formation as well as encouraging better cell attachment and activities. Our findings highlight that the use of K_2CO_3 had effectively enhanced the architecture and compressive strength of the CHA scaffolds without any toxicity evidence.

Keywords: Carbonated hydroxyapatite, Porous scaffold, Sintering aid, Replication technique, Biocompatibility

1. Introduction

Carbonated hydroxyapatite (CHA) has been considered as a better choice of bone material than hydroxyapatite (HA) for medical and dental applications. The presence of carbonate ions (CO_3^{2-}) in the apatite structure makes the composition more akin to the mineral content of native human bones and teeth [1], [2]. Although the amount of this ion is small (2-8 wt%), it is well known that CO_3^{2-} plays an essential role in bone metabolism [3], [4]. CHA can be classified into two types depending on the mode of carbonate (CO_3^{2-}) substitution either the hydroxyl (OH^-) or phosphate (PO_4^{3-}) ion positions in the apatite structure, forming A- or B-type CHA, respectively [5]–[7]. The formation of B-type CHA is the most preferred carbonate substitution as it is predominantly found in biological apatite, with the A/B type ratio in the range 0.7–0.9 [8].

Despite the excellent properties of CHA-based materials, it is known to have limited thermal stability. For instance, literature has shown that sintering of CHA-based materials in the air at high temperature (> 1000°C) led to total decomposition of carbonate from the CHA crystal structure, resulting in the formation of hydroxyapatite (HA) or calcium oxide (CaO) [9]. Typically, the decomposition of CHA begins at a low sintering temperature of about 600°C [10], [11]. This key challenge has remained over the years and hindered its clinical application as bone substitutes. The loss of carbonate ions from the CHA lattice could cause significant changes in the physico-chemical, mechanical and biological properties of the sintered CHA. Thus, careful control of the sintering profile (i.e. temperature, heating/cooling rate and atmosphere) is required to minimize the decomposition of carbonate from the CHA structure.

One of the typical approaches to overcome the problem of thermal instability of CHA is by introducing different sintering atmospheres (e.g. CO_2, O_2 or N_2). It has been reported that exposing B-type CHA in wet CO_2 atmosphere during sintering at high temperature favored in retaining high carbonate residue [9], [12]. However, from a materials engineering perspective,

this lengthy process at high temperature can be costly and detrimental to the interior lining of the furnace in the long run. Previously, our group had invented a simple and more economical technique where wet CO_2 gas was only pumped in during the cooling stage (at about 250°C) and yet had successfully produced single phase B-type CHA with up to 40% of CO_3^{2-} content retained in the apatite structure [13].

Alternatively, the incorporation of sintering additives or aids into the composition have also been suggested to improve the properties of the ceramic products. This additive material plays an essential role in reducing the sintering temperature and simultaneously enhance the densification of the ceramic [14]–[17]. The most commonly used sintering aids are alkaline-based oxide materials such as sodium, magnesium, calcium and potassium hydroxide or carbonate-based [18]. For instance, it has been reported that the use of a small amount (5-10 wt%) of sodium carbonate (Na_2CO_3) had significantly improved the microstructure and densification of HA pellets, which resulted in remarkable tensile strength, friability and disintegration time [19]. Other than Na_2CO_3, CHA with the addition of magnesium hydroxide, $Mg(OH)_2$ as sintering aid also produced dense structure with good mechanical properties and sufficient amount of carbonate remained [10].

A number of techniques have been developed for the fabrication of ceramic-based materials porous scaffolds, including polymeric foam replication, gel casting, pore forming agent, freeze drying and solvent casting/salt leaching [20], [21]. Polyurethane (PU) foam replication is a common technique for producing ceramic porous scaffold with a uniform interconnected pores structure as it exhibits a similar structure to the cancellous bone. This method offers scaffold designed with a wide range of pore size distribution having high porosity (60-90%) and allows various shapes/sizes of the scaffold to be produced [22]. Typically, synthetic polymers such as polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyacrylamide (PAA) are been used as binders in producing ceramic scaffolds by replication

technique [23], [24]. However, it has been reported that scaffolds produced using these binders either alone or in combination resulted in poor in densification and mechanical properties. Thus, it is necessary to explore the possibility of introducing other components into a ceramic slurry, including sintering aid and kaolin to improve the properties of developed CHA-based material scaffolds. In ceramic processing, kaolin is known to be "particle-reinforcing" of the structure due to its exceptional properties in which provides a good framework to the ceramic products. This is the rationale for its wide applications in technical porcelain and whiteware productions [25]. As far as the authors are aware, this is the first study introducing the combination of kaolin and sintering aid besides the use of binders in developing CHA porous scaffolds using polyurethane (PU) foam replication technique, thereby expecting an improvement of properties in terms of architecture, densification and mechanical.

Despite being recognized as a promising approach, majority of the studies reported focused only on the effect of sintering aids on the physical and mechanical properties of dense sintered ceramics. To our best of knowledge, the biological performances of these sintering aids, in particular on their *in vitro* bioactivity and biocompatibility properties are still lacking. To date, there is also a limited study on the role of sintering aid in making 3D CHA porous scaffolds found in the literature. Therefore, the focus of this work is to investigate the effectiveness of different types of sintering aids in producing interconnected 3D carbonated hydroxyapatite (CHA) porous scaffolds using PU replication technique. These scaffolds will then be characterized for their physico-chemical and mechanical properties followed by biological studies in order to determine their *in vitro* bioactivity and biocompatibility properties, which are crucial criteria for bone scaffolds. From a materials engineering point of view, we believe that the right choice of sintering aid plays an important role in determining the final properties of the produced porous scaffold, which includes the formation of B-type

CHA with a sufficient amount of carbonate as well as good architectural, mechanical and biological properties.

2. Materials and Methods

2.1 Fabrication of 3D CHA scaffolds

Carbonated hydroxyapatite (CHA) powder was chemically synthesized via nanoemulsion method at ambient temperature (27°C). The procedure to prepare as-synthesized CHA powders as described in our previous work reported elsewhere [26]. The powder produced was then used as the main raw material in preparing the 3D CHA scaffolds. These scaffolds were fabricated using polyurethane (PU) replication technique. Briefly, the CHA ceramic slurry was prepared by mixing the as-synthesized CHA powders with deionized (DI) water containing polyethylene glycol (PEG, M_w= 1500 g/mol: Fluka, Germany), polyvinyl alcohol (PVA, M_w= 15000 g/mol: Fluka, Germany), kaolin and dispersant (Dispex A40: BASF, Malaysia). A similar procedure was then applied in fabricating CHA porous scaffolds with different sintering aids namely, magnesium hydroxide, $Mg(OH)_2$, calcium hydroxide ($Ca(OH)_2$), sodium hydroxide (NaOH), potassium hydroxide (KOH) and potassium carbonate (K_2CO_3) as described above. All of these sintering aids were highly pure (> 90%) and purchased from Merck (Germany), except K_2CO_3 which was obtained from SYSTERM (Malaysia). The slurry prepared with viscosity of 71 Pa.s (Viscometer, Polyvisc Viscstar-H, GB) was then stirred for 1 hour at room temperature until a homogenous solution was achieved. Table 1 shows the amount of chemicals added to DI water to formulate the ceramic slurry.

PU foams (60 ppi) were cut into cubes with the dimension of 15 mm (length) × 15 mm (width) × 15 mm (thickness). These foams were subsequently dipped into the prepared CHA slurry followed by soaking for 5 minutes to allow sufficient impregnation of the slurry throughout the entire foam. The coated foams were then squeezed and released at least 10 times

to remove excess slurry before they were vacuumed and dried in an oven at 60°C for 24 hours. The dried coated foams were subsequently sintered in a chamber furnace (AWF-Laboratory Chamber Furnaces 1300°C, Lenton) at 800°C in air atmosphere. The sintered scaffolds were carefully removed from the furnace during the cooling stage (at 250°C) and directly placed in a desiccator. Wet carbon dioxide (CO_2) gas was immediately pumped into the desiccator at a rate of 0.5 L/min for 20 minutes to recompensate the loss of carbonate during sintering. Scaffolds were kept in desiccator for 24 hours before further characterizations were performed.

2.2 Material characterizations

X-ray Diffraction (XRD) analysis was carried out using a Bruker D8 XRD to determine the phase purity, crystallinity and lattice parameters of the sintered CHA scaffolds. XRD patterns were collected in the scan angles $20^{\circ} \leq (2\theta) \leq 70^{\circ}$ with a step size of 0.02° using Cu Kα radiation ($\lambda = 1.541$ Å), operating with PANalytical X'pert PRO diffractometer. Standard Hydroxyapatite (HA) with International Center for Diffraction Data (ICDD) file number of #09-0432 was used as the reference pattern. The type of bonding and mode of carbonate substitution (A-type or B-type) in the CHA structure were confirmed using Fourier Transform Infrared (FTIR) Spectroscopy. The samples were ground and mixed with potassium bromide (KBr), pelletized using a hand press with 5 MPa pressure and held for 2 minutes. The wavenumber was set in the range of 400.00 to 4000.00 cm^{-1}, scanned four times in transmittance mode (%T). Additionally, the amount of carbonate content present in the sintered scaffolds was determined using Carbon, Hydrogen, Nitrogen (CHN) Analysis (2400 Series II; Perkin-Elmer) in which the percentage of carbon obtained was multiplied by a factor of five.

The architecture of the internal struts for the sintered scaffolds was observed under Tabletop Scanning Electron Microscope (SEM) (TM3000, Hitachi). Prior to observation, each sample was coated with gold-palladium. The pore size of the porous scaffolds were then measured based on the SEM micrograph, where 20 readings were taken for each composition

in measuring the average pore size using ImageJ (v1.50i.). Compression test was performed using INSTRON 3369 universal tensile machine, employing the ASTM C1424-04: Standard test method commonly used for the advanced ceramics in terms of compressive strength, particularly at ambient temperature. For each composition, five scaffolds were tested and the average values of the compressive strength were then determined. The obtained values were then plotted as mean ± standard deviation. The *in vitro* bioactivity study of the sintered scaffolds was performed using simulated body fluid (SBF), prepared according to Kokubo's recipe [27]. The ability of apatite formation on the sintered scaffolds after immersion in SBF solution for 7, 14 and 21 days were examined using Field Emission Scanning Electron Microscope (FESEM, Zeiss Supra 55VP) equipped with Energy Dispersive X-ray (EDX). Each sample was coated with gold-palladium prior to the morphological observation to avoid the charging effect.

2.3 In vitro biocompatibility assessment

The International Organization for Standardization (ISO) 10993-5: Biological Evaluation of Medical Devices, Part 5: Test for Cytotoxicity, was adapted and used as guidelines in the biocompatibility assessment. This involved the study on cell viability, cell proliferation, total protein production and lactate release. *In vitro* biocompatibility was evaluated using human osteoblast-like cells (MG-63). The test was performed only on the optimum composition of the sintered CHA scaffolds. The main aim was to determine the level of toxicity of these compositions in direct contact with MG-63 cells. Sterilization was carried out by adding 1ml of 70% industrial methylated spirit (IMS) into the wells containing the respective samples and soaked overnight. Subsequently, the samples were then washed twice with phosphate-buffered saline (PBS) to prepare them for culture.

2.3.1 Culture and seeding

MG-63 cells at passage two (P2) were cultured in standard 4.5 g/L Dulbecco's Modified Eagle Medium (DMEM) containing 1% (v/v) L-glutamine (Lonza, United Kingdom), 1% (v/v) Penicilin-Streptomycin (Lonza, United Kingdom) and 10% (v/v) Fetal Bovine Serum (Biosera, United Kingdom). Cells were incubated at 37°C with 5% CO_2 and media was constantly replenished every three days until the required cell numbers were obtained. MG-63 cells at passage three (P3) were then seeded at 5 x 10^4 cells/tested samples. Cells were allowed to adhere overnight. Standard DMEM was used throughout the experiments. Samples for biochemical assays were collected on day 1 and 14. At each time point, cells were rinsed with PBS, then trypsinised and enzymatic digestion was performed overnight at 60°C. These cell lysates were then stored at -80°C until the assays were performed. Tissue culture plastic (TCP) was used as a positive control.

2.3.2 Cell viability

Cell viability and distribution on the tested samples were visualized under fluorescent microscope. Prior to observation, the cell-samples cultured at day 1 were initially stained using Live/Dead Kit (Invitrogen, United Kingdom) prepared according to the manufacturer's instructions. Calcein-AM ester was used to fluorescently stain viable cells in green, while Propidium Iodide was used to label the nucleus of the dead cells in red. At the time point, the cell culture media was removed followed by washing with 1 ml PBS. The samples were then stained using 0.5 ml PBS staining solution containing 10 μM Calcein-AM and 1 μM Propidium Iodide followed by incubation at 37°C with 5% CO_2 for 20 minutes in the dark. The samples (n = 1 for each composition) were then washed once using 1ml PBS and imaged under fluorescent microscope.

2.3.3 Cell proliferation

The cell proliferation in direct contact with the tested samples was evaluated using PicoGreen® DNA quantification assay (Quant-iTTM PicoGreen® dsDNA assay kit, Invitrogen, United Kingdom). Briefly, PicoGreen solution was prepared as 1: 200 dilutions in 1x Tris-EDTA (TE) buffer. The DNA standard curve was prepared using the following concentrations (0-1 μg/ml). An equal volume of cell lysate or DNA standard to PicoGreen reagent was placed into each well of a 96 well plate. The samples were then incubated for 5 minutes in the dark, prior reading the fluorescence at 485/535 nm.

2.3.4 Total protein production

The levels of total protein were quantified using Bradford reagent (Sigma-Aldrich, United Kingdom). Ranges of protein standard solutions (0-2 mg/ml) were prepared by dissolving Bovine Serum Albumin, BSA (Sigma-Aldrich, United Kingdom) in distilled water. For total protein assay, equal amounts of cell lysates or standard solution and Bradford reagent were placed in each well of 96 well plates. They were incubated for 5 minutes at room temperature before reading the absorbance level at 595 nm.

2.3.5 *Cytotoxicity*

Lactate dehydrogenase (LDH) assay is a reliable colorimetric assay to the amount of lactate excreted into the culture medium from damaged cells as a biomarker for cellular cytotoxicity. LDH kit (Pierce LDH Cytotoxicity Assay Kit, ThermoScientific) was used according to manufacturer's instruction. The cell culture media were collected at two time points (1 and 14 days) and kept at -21°C. A minimum of $n = 5$ readings were taken for each time point. LDH activity was expressed as absorbance (OD) measured at 490/ 680 nm (excitation/ emission).

2.3.5 Statistical Analysis

Quantitative data for biochemical assays were presented as means ± standard deviation (SD). Data were tested using two ways ANOVA with Tukey test to determine the optimum composition between the tested samples. The optimum composition refers to samples that provide a favorable environment for cell attachment, encourage the highest level of cell proliferation and total protein production as well as cyto-compatibility. Statistical significance was considered for $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0001$ (****). Tests were performed on $n = 3$ in duplicate for biochemical assays and $n = 1$ for live/dead staining. GraphPad Prism software version 7.0 was used to conduct statistical analyses.

3. Results and Discussion

3.1 XRD analysis

XRD patterns of the sintered CHA scaffolds fabricated using different sintering aids in comparison to CHA scaffold without sintering aid are shown in Fig. 1. The diffraction patterns of all the sintered CHA scaffolds remained as a single phase of HA (ICDD #09-0432) with a slight shift due to the carbonate substitution into the apatite structure. There was no secondary phase such as $CaCO_3$ (ICDD #01-1032) or CaO (ICDD #03-0865) appeared in any of the diffraction patterns. It was confirmed that the CHA scaffolds sintered at 800°C with or without sintering aid did not cause any formation of other phases and remained the structural integrity of CHA. The sintered scaffolds showed high degree of crystallinity as represented by the sharp and narrow diffraction peaks. Nine prominent peaks were detected in the range of $20^{\circ} \leq (2\theta) \leq 70^{\circ}$, with three main overlapping peaks at 2θ = 31-34˚, represented by the reflections of (211), (300) and (202). Other planes such as (002), (130), (222), (213) and (004) matched with HA pattern were also detected for all sintered CHA scaffolds [11], [28]. A minor peak of (112) at $2\theta = 33^{\circ}$ was also observed; in typical CHA pattern, the (112) peak is not observed. In this work, the (112) peak suggests the onset of carbonate decomposition due to the sintering performed. However, it will later be confirmed using FTIR and CHN analyses.

Generally, the sintered scaffolds showed expansions in both of the lattice parameters in comparison to the standards reported in the ICDD (Table 2). The CO_3^{2-} substitution into the apatite structure led to the alteration in the crystal lattice parameters [6], [29]. This can be explained whereby the substitution of small planar trigonal CO_3^{2-} group into the large planar tetrahedral PO_4^{3-} group, thus resulting in a contraction of *a*-axis and expansion of the *c*-axis [30]. However, the trigonal planar CO_3^{2-} group could also be partially substituted into smaller Ca^{2+} site, causing the expansion of both *a*- and *c*-axes as CO_3^{2-} simultaneously substituted into both Ca^{2+} and PO_4^{3-} sites. A similar trend was previously reported by our group [31].

3.2 FTIR analysis

The FTIR spectra of the sintered CHA scaffolds with various sintering aids in comparison to CHA scaffold without sintering aid are presented in Fig. 2. All the sintered scaffolds exhibited the characteristic bands of the phosphate groups at about 465-475 cm^{-1} (v_2), 550-570 and 600-609 cm^{-1} (v_4), 960-966 cm^{-1} (v_1), and 1020-1120 cm^{-1} (v_3) [4], [32]. The presence of both absorbed and occluded water were also detected at 3400-3600 cm^{-1} and 1600-1700 cm^{-1}, representing the OH^- group [33].

The presence of carbonate in the apatite structure can be seen in the spectra for all the sintered scaffolds. Three typical bands of B-type CHA originating from stretching vibration of CO_3^{2-} at around 870-875 cm^{-1}, 1412-1430 and 1450-1470 cm^{-1} were detected. The result confirms that the sintered scaffolds are of B-type CHA upon sintering at 800°C. Similar trends were previously reported in the literature [34], [35]. The typical bands for A-type CHA, which usually appear at 877-880, 1500 and 1540-1545 cm^{-1} were not visible in this work [10], [36]. However, there is a slight decrease in the intensity of the CO_3^{2-} groups, particularly at 1412-1430 and 1450-1470 cm^{-1}. This suggests an early stage of CO_3^{2-} decomposition, thus explaining the detection of peak (112) in the XRD pattern as reported earlier. According to literature,

CO_3^{2-} normally start to decompose at low sintering temperature of about 600°C. This is witnessed by the flattens curve of CO_3^{2-} bands for CHA scaffold without sintering aid. Thus, our result leads to two important findings: (1) the use of sintering aids had effectively reduced the sintering temperature without causing any changes to the carbonate substitution of the apatite and (2) the introduction of wet CO_2 during the cooling stage had recompensated the carbonate loss upon sintering.

3.3 CHN Analysis

The amount of carbonate retained in all the sintered CHA scaffolds was about 2.44-3.40 wt% (Table 2). These amounts fall in the range of carbonate content (2-8 wt%) that is typically present in human bone mineral [11], [35]. Despite of the carbonate loss, sufficient amount of carbonate could still be retained even by sintering the scaffolds at 800°C. More importantly, the used of sintering aid had successfully retained up to 3-4-folds carbonate content as compared to CHA scaffold prepared without sintering aid. With regards to the aforementioned weakness of the CHA sintered scaffold without sintering aid, the following discussion will focus only on CHA scaffolds prepared with various sintering aids in order to determine the most effective one. The CHN result obtained supports the FTIR observation.

3.4 SEM Observation

Regardless of the sintering aid used, the average pore sizes obtained for all the sintered scaffolds were in the range of 290-490 μm which falls in the range of the required pore size for ideal bone scaffolds (200-500 μm). Several studies had also demonstrated that bone scaffolds with interconnected pore structure and pore sizes > 300 μm could support better osteogenesis

(bone formation), as it is suitable for cell migration, vascularization and nutrient diffusion [37]–[40].

Interestingly, the addition of different sintering aids had influenced the architecture of the sintered CHA scaffolds (Fig. 3). Among the sintered scaffolds, a more uniform open pore structure was observed for CHAMg and CHAKC scaffolds, indicating the effectiveness of $Mg(OH)_2$ and K_2CO_3, respectively as a sintering aid. Besides having uniform open pores structure, both sintered scaffolds also revealed good interconnected pores and good strut features with minimum cracks as compared to the scaffolds prepared using other sintering aids. Even at low sintering temperature (800°C), the use of $Mg(OH)_2$ and K_2CO_3 as sintering aids were able to induce sufficient densification. According to the literature, the formation of this liquid phase through the Liquid Phase Sintering (LPS) could provide better rearrangement of ceramic particles and enhance matter transport, which leads to the densification of the green ceramic body during the initial and middle stages of sintering. Thus, with the addition of 5wt% $Mg(OH)_2$ or K_2CO_3 into the CHA ceramic slurry, a transient liquid phase was introduced which then filled in the gaps and created better bonding between the CHA nano-sized particles. This had enhanced the densification of the struts during sintering. Besides, it is also observed that the addition of kaolin as a reinforcing agent into ceramic slurry had enhanced its densification and physical performance to a better extent in terms of structure and rigidity of the sintered scaffold. In our preliminary study, it was found that without the addition of kaolin into the slurry, the scaffolds were totally collapsed after sintering.

In contrast, the use of $Ca(OH)_2$, NaOH and KOH as CHACa, CHANa and CHAKO scaffolds demonstrated that these sintering aids had negligible effect on the densification of sintered CHA scaffolds. These sintering aid caused clogging which can be clearly seen for CHACa, CHANa and CHAKO sintered scaffolds. Cracks were also formed on the struts. This could possibly be attributed to the sintering aids being ineffective enough to provide liquid

phase formation. Hence, the particles were not well bound to each other in order to induce sufficient densification during sintering. It can be reported that the addition of $Mg(OH)_2$ (CHAMg scaffold) or K_2CO_3 (CHAKC scaffold) as the sintering aid showed optimal ceramic slurry composition resulting in scaffolds having well interconnected pores with densified struts, easy to handle and provide sufficient compressive strength.

3.5 Compressive strength

Compressive strength of the sintered scaffolds was determined on the different scaffolds using the various sintering aids (Fig. 4). This clearly highlighted the role of sintering aid in densification, particularly on the struts of the sintered scaffolds, which directly correlates to the compressive strength of the scaffolds. Among the sintered scaffolds, CHAKC exhibited the highest compressive strength of about 0.50 ± 0.05 MPa, followed closely by CHAMg scaffold (0.37 ± 0.10 MPa). Both of these scaffolds are comparable to the minimum limit of natural cancellous bone strength (0.10 to 16.00 MPa) [41]. Moreover, these two scaffolds provide sufficient mechanical strength, which allow the ease of handling. This is an important criteria for ideal scaffold to be used in clinical particularly during implantation and remain steadily during the recovery process of the patient [42]. However at this point, further studies in producing 3D CHA scaffolds are required in order to improve the mechanical strength of these scaffold materials as bone substitutes in clinical applications.

The mechanical strength of CHA scaffolds showed a consistent trend with the microstructural observations, where both of these porous scaffolds showed open and interconnected pores as well as more densified struts (Fig. 3). Clearly, the presence of clogged or blocked pores observed on CHACa, CHANa and CHAKO resulted in lower compressive strength values. Despite the compositions of the scaffolds, the average total porosity of the scaffolds produced lies between 83 to 92%, which is suitable for ideal bone scaffolds as described in the literature [43]. It is well understood that bone scaffolds with higher density

would lead to higher mechanical strength while a highly porous structure provides lower mechanical strength but a more favorable biological environment for cell growth and activities including cell attachment, proliferation and differentiation. Thus, a balance in porosity is required in fabricating CHA porous scaffolds with good mechanical and biological performances [44]. From the results obtained, CHAMg and CHAKC can be reported as the optimum compositions in producing the sintered CHA scaffolds. However, producing bone scaffolds with suitable physico-chemical and mechanical properties alone is insufficient without considering the biological effect of these materials [45], [46]. Therefore, it is important to ensure that these scaffold materials are biocompatible and able to encourage cell growth and activities.

3.6 In vitro bioactivity evaluation

Fig. 5 shows the FESEM micrographs of the selected sintered scaffolds (CHAMg and CHAKC) after been immersed in simulated body fluid (SBF) for 21 days. Early formation of apatite precipitates were observed on the surface of CHAMg and CHAKC scaffolds, after 7 days of immersion. By prolonged the immersion period up to 14 days, a well-dispersed flower-like apatite layer was observed on the surface of CHAKC scaffold. A further increase in the formation of the apatite layer was observed on both CHAMg and CHAKC scaffolds as the immersion time was prolonged to 21 days. In general, larger area of flower-like apatite was found on the surface of CHAKC scaffold in comparison to CHAMg scaffold. This is probably due to the interconnected structure with fully densified struts of CHAKC scaffold, which provides a more favorable environment for the formation of the apatite layer. It is well documented that the characteristic structure of biomaterial scaffolds such as suitable pore size, sufficient of porosity and well interconnected pores structure are crucial factors, in offering better biological delivery throughout the entire scaffolds [47]. The Ca/P ratio of amorphous precipitate also increased (> 1.67) proportionally with the immersion time in SBF solution (data

not shown for brevity), thus confirming that the precipitates are calcium phosphate apatite crystals [48], [49]. This result suggests that the addition of K_2CO_3 as sintering aid into the slurry composition (CHAKC scaffold) had provided suitable physico-chemical and mechanical properties as well as improved the bioactivity of the sintered scaffold.

3.7 In vitro Biocompatibility Assessment

Toxicity of material in direct contact with the biological cells is mainly due to either the release of toxic ions compounds or worn debris from the ceramic material itself [42], [49], [50]. The early sign of toxicity of a material can be clearly seen by the changes of cell morphology, level of DNA concentration, total protein production and cytotoxicity by lactate release [51].

In this study, the viability of MG-63 cells seeded on CHAMg and CHAKC samples were observed after 1 day of culture and compared to the tissue culture plastic (TCP), which served as positive control (Fig. 6). Generally, it can be observed that the MG-63 cells remained viable when in direct contact with both tested samples and showed a similar trend as the control TCP, indicating that both compositions are biocompatible. MG-63 cells showed elongated fibroblasts-like morphology which almost covered the entire surface of the tested samples. In particular, CHAKC was found to provide the more favorable environment for the growth and activities of MG-63, as more viable cells (green fluorescent stain) were observed in comparison to CHAMg, which showed a relatively higher proportion of dead cells (red fluorescent stain).

The proliferation of MG-63 seeded on CHAMg and CHAKC was assessed by their level of DNA after day 1 and 14 in culture (Fig. 7). The results obtained on day 1 is consistent with the cell viability shown in the Live/Dead images (Fig. 6). At this early stage of culture, both compositions of the tested samples showed low level of DNA concentration with no significant differences (p = 0.1084) observed. As the culture was prolonged to 14 days, CHAKC demonstrated a significantly higher level of DNA concentration (p = 0.0146)

compared to CHAMg. Generally, both CHAMg and CHAKC are able to encourage cell proliferation in direct contact with MG-63 cells. This highlight that the use of both sintering aids, namely $Mg(OH)_2$ and K_2CO_3 into the CHA ceramic slurry in making the scaffolds does not cause any toxicity effect to the cells.

In spite of the composition, both CHAMg and CHAKC tested samples also demonstrated similar level of total protein production on day 1 (Fig. 8). At this early time point, CHAMg and CHAKC showed significantly lower levels of total protein produced as compared to the control sample (p = 0.0226 and p = 0.0179, respectively). Over time, the level of total protein production gradually increased for both tested samples. At day 14, CHAKC produced a slightly higher level of total protein as compared to CHAMg (p = 0.9957). This supports the previous observation, where cells grown on CHAKC showed better viability and proliferation. It has been emphasized in literature that the presence of carbonate in the apatite ceramic material resulted in higher metabolic activity of the particular cells/tissues [36], [51]. The results obtained thus for suggested that the use of $Mg(OH)_2$ or K_2CO_3 as sintering aids as well as decarbonation during the sintering cooling stage had successfully retained sufficient amount of carbonate content needed to encourage cell proliferation and total protein production. Protein is an important building block particularly for cell attachment, proliferation and differentiation. Besides collagenous material, which is the major protein found in the extracellular matrix (ECM) of human native bone; there are over 200 different types of non-collagenous proteins presence in bone ECM. However, at this stage of the study the type of protein produced has not been determined as yet.

The LDH released by MG-63 cells cultured on CHAMg and CHAKC tested samples was assessed as an indication of cytotoxicity mediated by chemical compounds (Fig. 9). It has been shown that both tested samples did not exhibit any cytotoxic effect with respect to control TCP up to 14 days. The highest amount of LDH released was recorded for CHAMg, followed

by CHAKC and control TCP at day 1 ($p \leq 0.0001$). This is well correlated with the observation on cell viability, where lower proportion of viable cells were found on CHAMg as compared to CHAKC at the early period of culture (Fig. 6). The continuous rise of LDH activity can be observed with prolonged periods of time (14 days), where the amount of LDH released for CHAMg is significantly higher as compared to CHAKC ($p \leq 0.0001$). On the other hand, the high LDH released of the control TCP on day 14 is expected due to over confluence of the cells. Overall, the LDH activity for both tested samples showed a consistent trend with cell viability, cell proliferation and the level of total protein obtained.

4. Conclusion

Three-dimensional (3D) carbonated hydroxyapatite (CHA) porous scaffolds were successfully fabricated using polyurethane (PU) replication technique. The presence of sintering aid in the system had produced 3D interconnected porous scaffolds with single phase B-type CHA and improved amount of carbonate retained in structure as compared to CHA scaffold prepared without sintering aid. Amongst the five tested sintering aids, K_2CO_3 was found to be the most effective sintering aid, resulting in the most densified struts with less cracks as well as having open interconnected pores with suitable pores sizes as required for ideal bone scaffolds. These features had enhanced the compressive strength of the fabricated scaffolds and provided better handling compared to other prepared scaffolds. Interestingly, CHA porous scaffolds prepared by the addition of K_2CO_3 demonstrated good bioactivity and biocompatibility properties. However, further *in vitro* studies such as cell attachment and differentiation will be carried out in the future to provide in-depth understanding on cell activities in direct contact with these fabricated scaffolds. Thus far, our findings suggest that K_2CO_3 is the most promising sintering aid in producing 3D CHA scaffolds; this piece of information might be of great help to other researchers working in this particular area. In the

near future, various amount of K_2CO_3 will be investigated to enhance further the performance of 3D CHA porous scaffolds in bringing this bone substitute closer to the clinical applications.

Conflict of Interest

The authors declare no conflict of interest.

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References

[1] Sprio S., Tampieri A., Landi E., Sandri M., Martorana S., Celotti G. and Logroscino G., Physico-chemical properties and solubility behaviour of multi-substituted hydroxyapatite powders containing silicon, *Mater. Sci. Eng. C.* 28 (1) (2008) 179-187.

[2] Wang K., Wang Y., Zhao X., Li Y., Yang T., Zhang X., & Wu X., Sustained release of simvastatin from hollow carbonated hydroxyapatite microspheres prepared by aspartic acid and sodium dodecyl sulfate, *Mater. Sci. Eng. C.* 75 (2017) 565-571.

[3] Marković S., Veselinović L., Lukić M. J., Karanović L., Bračko I., Ignjatović N., & Uskoković D., Synthetical bone-like and biological hydroxyapatites: a comparative study of crystal structure and morphology, *Biomed. Mater*. 6 (4) (2011) 045005.

[4] Yang W. H., Xi X. F., Li J. F., & Cai K. Y., Comparison of crystal structure between carbonated hydroxyapatite and natural bone apatite with theoretical calculation, *Asian J. Chem.* 25 (7) (2013) 3673–3678.

[5] Zyman Z. and Tkachenko M., CO_2 gas-activated sintering of carbonated hydroxyapatites, *J. Eur. Ceram. Soc.* 31 (3) (2011) 241–248.

[6] Bang L.T., Long B.D. and Othman R., Carbonate hydroxyapatite and silicon-substituted carbonate hydroxyapatite: synthesis, mechanical properties, and solubility evaluations, *Sci. World J*. (2014).

[7] Rajabi-Zamani A. H., Behnamghader A., & Kazemzadeh A., Synthesis of nanocrystalline carbonated hydroxyapatite powder via nonalkoxide sol–gel method, *Mater. Sci. Eng. C.* 28 (8) (2008) 1326-1329.

[8] Kee C. C., Ismail H., & Ahmad-Fauzi M.N., Effect of synthesis technique and carbonate content on the crystallinity and morphology of carbonated hydroxyapatite, *J. Mater. Sci. Technol.* 29 (8) (2013) 761–764.

[9] Barralet J., Knowles J.C., Best S. and Bonfield W., Thermal decomposition of synthesised carbonate hydroxyapatite, *J. Mater Sci.: Mater. Med.* 13 (6) (2002) 529-533.

[10] Yanny Marliana B.I and Ahmad-Fauzi M.N., Effect of a novel approach of sintering on physical properties of carbonated hydroxyapatite. *J. Mater. Sci. Eng. B*. 1 (2) (2011) 157–163.

[11] Safarzadeh M., Ramesh S., Tan C. Y., Chandran H., Ching Y. C., Ahmad-Fauzi M.N. & Teng W. D., Sintering behaviour of carbonated hydroxyapatite prepared at different carbonate and phosphate ratios. *Boletín de la Sociedad Española de Cerámica y Vidrio*, 59 (2) (2020) 73-80.

[12] Barinov S. M., Rau J. V., Cesaro S. N., Ďurišin J., Fadeeva I. V., Ferro D., & Trionfetti G., Carbonate release from carbonated hydroxyapatite in the wide temperature rage, *J. Mater. Scie. Mater. Medo.* 17(7) (2006) 597-604.

[13] Wong, W. Y. and Ahmad-Fauzi M.N., Synthesis and Sintering-wet Carbonation of Nano-sized Carbonated Hydroxyapatite, *Procedia Chem.* 19 (2016) 98–105.

[14] Soto C., García-Rosales C. and Echeberria J., Production of porous SiC by liquid phase sintering using graphite as sacrificial phase: Influence of SiO_2 and graphite on the sintering mechanisms, *J. Eur. Ceram. Soc.* 39 (14) (2019) 3949–3958.

[15] Demirkiran H., Mohandas A., Dohi M., Fuentes A., Nguyen K., & Aswath P., Bioactivity and mineralization of hydroxyapatite with bioglass as sintering aid and bioceramics with $Na_3Ca_6(PO_4)_5$ and $Ca_5(PO_4)2SiO_4$ in a silicate matrix, *Mater. Sci. Eng. C. 30*(2) (2010) 263-272.

[16] Ge L., Gu Y., Zhang Y., Li X., Ni Q., & Guo L., Effect of sintering aids on the densification and electrical properties of SiO^{2-}containing $CeO.8SmO.2O_{1.9}$ ceramic, *J. Eur. Ceram. Soc.* 38 (6) (2018) 2553-2561.

[17] Jiang G. P., Yang J. F., Gao J. Q., & Niihara K., Characterization of porous silicon nitride ceramics using bentonite as binder and sintering additive, *Mater. Charact.* 60 (5) (2009) 456-460.

[18] Suchanek W., Yashima M., Kakihana M., & Yoshimura M., Hydroxyapatite ceramics with selected sintering additives, *Biomaterials*. 18 (13) (1997) 923–933.

[19] Partheniadis I., Papanikolaou T., Noisternig M. F., Griesser U. J., Kantiranis N., & Nikolakakis I., Structure reinforcement of porous hydroxyapatite pellets using sodium carbonate as sintering aid: Microstructure, secondary phases and mechanical properties, *Adv. Powder Technol*. 30 (8) (2019) 1642-1654.

[20] Pia G., Casnedi L. and Sanna U, Porous ceramic materials by pore-forming agent method : An intermingled fractal units analysis and procedure to predict thermal conductivity, *Ceram. Int.* 41 (5) (2015) 6350–6357.

[21] Asefnejad A., Khorasani M.T., Behnamghader A., Farsadzadeh B. and Bonakdar S., Manufacturing of biodegradable polyurethane scaffolds based on polycaprolactone using a phase separation method: physical properties and in vitro assay., *Int. J. Nanomedicine*. 6 (2011) 2375–2384.

[22] Da Mota M., De V. and Branco A., Polyurethane-based Scaffolds for Bone Tissue Regeneration. 1 (2015) 1–10.

[23] Saber-Samandari S., Ghonjizade-Samani F., Aghazadeh J., & Sadeghi A., Bioactivity evaluation of novel nanocomposite scaffolds for bone tissue engineering: The impact of hydroxyapatite, *Ceram. Int.* 42 (9) (2016) 11055-11062.

[24] Monmaturapoj N., & Yatongchai C., Influence of preparation method on hydroxyapatite porous scaffolds, *Bull. Mater. Sci.* 34 (7) (2011) 1733–1737.

[25] Štubňa I., Šín P., Trník A., & Veinthal R., Mechanical properties of kaolin during heating. *Key Eng.Mater.* 527 (2013) 14-19.

[26] Muhammad Syazwan M.N. and Yanny Marliana B.I., The influence of simultaneous divalent cations (Mg^{2+}, Co^{2+} and Sr^{2+}) substitution on the physico-chemical properties of carbonated hydroxyapatite, *Ceram. Int*. 45 (12) (2019) 14783-14788.

[27] Kokubo T., & Takadama H., How useful is SBF in predicting in vivo bone bioactivity?, *Biomaterials*. 27 (15) (2006) 2907–2915.

[28] Robert C., Darshana D., and Richard P., Bone Remodeling, *Encycl. Br.* (2012).

[29] Kovaleva E. S., Shabanov M. P., Putlayev V. I., Filippov Y. Y., Tretyakov Y. D., & Ivanov V. K. Carbonated hydroxyapatite nanopowders for preparation of bioresorbable

[30] Wopenka B., & Pasteris J. D., A mineralogical perspective on the apatite in bone, *Mater. Sci. Eng. C*. 25 (2) (2005) 131–143.

[31] Ezekiel I., Shah Rizal K., Yanny Marliana B.I., and Ahmad-Fauzi M.N., Nanoemulsion synthesis of carbonated hydroxyapatite nanopowders : Effect of variant CO_3^{2-} / PO_4^{3-} molar ratios on phase, morphology, and bioactivity, *Ceram. Int.* 44 (11) (2018) 13082–13089.

[32] Mondal B., Mondal S., Mondal A., & Mandal N., Fish scale derived hydroxyapatite scaffold for bone tissue engineering, *Mater. Charact.* 121 (2016) 112-124.

[33] Hazar Yoruc A. B., Karakaş A., Ayas E., & Koyun A., Effect of precipitation method on properties of hydroxyapatite powders, *Acta Phys. Pol. A.* 123 (2) (2013) 371–373.

[34] Kumar G. S., Thamizhavel A., Yokogawa Y., Kalkura S. N., & Girija E. K., Synthesis, characterization and in vitro studies of zinc and carbonate co-substituted nano-hydroxyapatite for biomedical applications, *Mater. Chem. Phys*. 134 (2–3) (2012) 1127–1135.

[35] Januariyasa I. K., Ana I. D., & Yusuf Y., Nanofibrous poly (vinyl alcohol)/chitosan contained carbonated hydroxyapatite nanoparticles scaffold for bone tissue engineering, *Mater. Sci. Eng. C*. 107 (2020) 110347, 2020.

[36] Landi E., Celotti G., Logroscino G., & Tampieri A., Carbonated hydroxyapatite as bone substitute, *J. Eur. Ceram. Soc.* 23 (15) (2003) 2931–2937.

[37] Dorozhkin S. V., Calcium Orthophosphate ($CaPO_4$) Scaffolds For Bone Tissue Engineering Applications, *J. Biotechnol. Biomed. Sci*. 1 (3) (2018) 25.

[38] Youness R. A., Taha M. A., & Ibrahim M. A., *In vitro* bioactivity, molecular structure and mechanical properties of zirconia-carbonated hydroxyapatite nanobiocomposites sintered at different temperatures, *Mater. Chem. Phys.* 239 (2020) 122011.

[39] Fiume E., Barberi J., Verné E., & Baino F., Bioactive glasses: from parent 45S5 composition to scaffold-assisted tissue-healing therapies, *J. Funct. Biomater.* 9 (1) (2018) 24.

[40] Wu S. C., Hsu H. C., Hsu S. K., Wang W. H., & Ho W. F., Preparation and characterization of four different compositions of calcium phosphate scaffolds for bone tissue engineering, *Mater. Charact.* 62 (5) (2011) 526-534.

[41] Gerhardt L.C. and Boccaccini A.R., Bioactive glass and glass-ceramic scaffolds for bone tissue engineering. *Materials*. 3 (7) (2010) 3867-3910.

[42] Schwartz Z., Raz P., Zhao G., Barak Y., Tauber M., Yao H. and Boyan B.D., Effect of micrometer-scale roughness of the surface of Ti_6Al_4V pedicle screws *in vitro* and *in vivo*, *J. Bone Jt. Surg.* 90 (11) (2008) 2485.

[43] Pilia M., Guda T. and Appleford M., Development of composite scaffolds for load-bearing segmental bone defects, *BioMed Res. Int*. (2013).

[44] Jamaludin A.R., Shah Rizal K., Ismail A.K., Abdullah M.Z. and Ahmad Z.A, The effect of sago as binder in the fabrication of alumina foam through the polymeric sponge replication technique, *J. Eur. Ceram. Soc.* 35 (6) (2015) 1905–1914.

[45] Sabree I., Gough J. E., & Derby B., Mechanical properties of porous ceramic scaffolds: Influence of internal dimensions, *Ceram. Int.* 41 (7) (2015) 8425–8432.

[46] Asa'ad F., Pagni G., Pilipchuk S. P., Giannì A. B., Giannobile W. V., & Rasperini G., 3D-printed scaffolds and biomaterials: review of alveolar bone augmentation and periodontal regeneration applications, *Int. J. Dent*. (2016).

[47] Zhu L., Luo D., & Liu Y., Effect of the nano/microscale structure of biomaterial scaffolds on bone regeneration, *Int. J. Oral Sci.* 12 (1) (2020) 1-15.

[48] Czikó M., Bogya E.S., Barabás R., Bizo L. and Stefan R., In vitro biological activity comparison of some hydroxyapatite-based composite materials using simulated body fluid, *Cent. Eur. J. Chem*. 11 (10) (2013) 1583-1598.

[49] Settanni G., Zhou J., Suo T., Schöttler S., Landfester K., Schmid F. and Mailänder V., Protein corona composition of PEGylated nanoparticles correlates strongly with amino acid composition of protein surface, *arXiv preprint arXiv.* (2016) 1612.08814.

[50] Rupani A., Balint R. and Cartmell S.H., Osteoblasts and their applications in bone tissue engineering, *Cell Health Cytoskeleton*. 4 (2012) 49-61.

[51] Yanny Marliana B.I., Wimpenny, O. Bretcanu, K. Dalgarno, and A. J. El Haj, Development of multisubstituted hydroxyapatite nanopowders as biomedical materials for bone tissue engineering applications, *J. Biomed. Mater. Res. - Part A*. (2017) 1–1.