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Characterization of carbonate apatite derived from chicken bone and its in-vitro evaluation using MC3T3-E1 cells

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Abstract

Chicken bone by-product has the potential to be utilized as a source of carbonate apatite (CO3Ap) for the preparation of bone grafts. In this study, the raw bones were cleaned with NaCl and then immersed in NaOH solution at 80 °C. After chemical treatment, the as-prepared powder was calcined in a range of 300 °C–900 °C. The results showed that calcination was useful for the complete elimination of organic residues; but, it led to increasing of crystal size, coarsening of particles, and the loss of the carbonate groups in the apatite structure. In vitro evaluation showed that CO3Ap prepared at the optimum temperature, 500 °C, exhibited a great biological response; such as good cell attachment, cell viability, and cell morphology. This research suggests a promising biomaterial for the fabrication of bone substitutes and could offer a solution for recycling and adding value to food waste.

Introduction

Every year, many by-product materials from animal agriculture and food industry are produced globally. Wastes such as seafood shells, eggshells, poultry and animal bones are frequently discarded in a landfill or simply dumped into the ocean. The environmental issues also happened due to improper management, consequently, the financial costs for solving the pollutants are substantial. One challenge to this is that the potential value of the waste is not considered high enough to the other fields. Therefore, it is necessary to conduct more research to turn this abundant source into value-added products.

In biomedical applications, the inorganic content of poultry and animal bones is mainly carbonate apatite (CO3Ap) that could be used as the raw material for fabrication of bone grafts. Components of the natural bone were basically 60%–70% of inorganic minerals, 20%–30% of collagen and other organic components. The inorganic component of natural bone is known as bone apatite, which contains calcium and phosphate as the dominant components. Bone apatite also contains a majority of carbonate (4–8 mass %) and other elements and is, therefore, classified as carbonate apatite (CO3Ap: Ca10-(PO4)6−b(CO3)c(OH)2) [1, 2]. Bone grafts are one of the prospective bone substitutes for treatment of bone-related diseases, skeletal defects, tumor resection, skeletal abnormalities and bone fractures from trauma. A bone graft is an alternative solution for bone defect treatment because the use of natural bone grafts has many disadvantages such as limited supply, trauma and additional pain for patients. CO3Ap as artificial bone substitute proved good osteoconductive and osteogenic properties in many studies [3–5].

Previous techniques such as precipitation, hydrothermal and mechanical activation were used to synthesize CO3Ap powder [6–12]. Driven by environmental and economic consideration, apatite powder has also been extracted from natural bone sources such as pig and cow bones or teeth, etc. This method is advantageous on economic and environmental senses since it used the waste products of food industry as the raw materials [13–16].
In this study, waste chicken bone was used as raw material to extract CO₃Ap powder. It is expected to offer a solution for recycling and adding value to the food by-product. In order to obtain the required properties of powder for fabrication of bone graft, heat treatment is necessary to form the rigid blocks or granules. For implantation, a granule, block or cement form must be used to prevent inflammation since powder could detach and disperse easily to human body fluid. Since the source of this is CO₃Ap powder is waste chicken bone, heat treatment is also needed for removing completely organic residue. Therefore, in this study, raw chicken bone powder was calcined to different temperatures ranging from 300 °C–900 °C for characterization. Since processing of the CO₃Ap powder into rigid shape for bone graft using heat treatment generally affects the cellular response properties, the in vitro evaluation using the osteoblastic MC3T3-E1 cells was conducted. Gaining insight into the interactions between heat-treated CO₃Ap derived from waste chicken bone and the osteoblastic MC3T3-E1 cells could provide essential information about the biological properties of this recycled material and its potential use as a biomaterial for bone repair.

Materials and method

Preparation
Chicken bone was collected from food waste. Meat and fat residues were removed first by mechanical processes and the remaining material was then rinsed with distilled water. The raw bone was then immersed in 2% sodium chloride solution (NaCl, 7548–4100) for 2 h at 100 °C in order to further remove other debris.

The bone was then rinsed with distilled water and immersed in 2 mol l⁻¹ sodium hydroxide solution (NaOH, 7571–4400) for 12 h at 80 °C. NaCl and NaOH were purchased from Daejung Chemical & Metals Co. Ltd., Gyeonggi-do, Korea. Finally, the bone material was washed and filtered several times with distilled water, using a vacuum pump, until the filtrate was neutral.

The as-prepared bone was dried in the oven for 24 h and ground until the powder can pass through the 45 μm sieve. The as-prepared powders were then dried in the oven and calcined to different temperatures ranging from 300 °C–900 °C for 2 h in the furnace (ARF-30MC, Asahi Rika Seisakusho Co., Ltd., Chiba, Japan).

Phase characterization
Phase composition characterization was done by means of powder x-ray diffraction (XRD) analysis. The XRD patterns were recorded using a diffractometer system (D8 Advance, Bruker AXS GmbH, Karlsruhe, Germany) using Vario1 Johansson focusing mono-chromator and high flux CuKα radiation generated at 40 kV and 40 mA. The specimens were scanned from 2θ = 20 ° to 60 ° (where θ is the Bragg angle) in a continuous mode. The location of the carbonate group in the apatite structure was evaluated using Fourier Transform Infrared Spectrometer (FTIR). Carbonate content of the specimens was evaluated using CHN coder (Yanako CHN coder. MT-6, Tokyo, Japan).

Thermal properties
Thermal properties were determined via Thermogravimetry (TG) and Differential Scanning Calorimetry (DSC) techniques (Netzsch STA 449 F5 Jupiter, NETZSCH–Gerätebau GmbH, Wittelsbacherstraße, 42 95100 Selb, Germany). The specimen was heated from room temperature to 1000 °C with heating rate of 20 K min⁻¹.

Microstructure properties
The size and shape of the powder particles were observed using a Transmission Electron Microscope (TEM: JEM-1400, JEOL, Japan). As-prepared and 500 °C-calcined CO₃Ap powders were dispersed in ethanol. After that, each suspension was sonicated for 30 min at high power. Then, 3 ml of each sample were dropped onto a copper grid and dried in air for 5 min before being inserted into the microscope.

In vitro cell culture
To study the biological properties of bone apatite, the as-prepared powder was pressed unaxially by an oil press machine (Riken Power, Riken Seiki, Tokyo, Japan) at 40 MPa to obtain a block-type specimens. The obtained blocks were placed in a ceramic electric tubular furnace (ARF-30MC, Asahi Rika Seisakusho Co., Ltd., Chiba, Japan) and heated from room temperature to 500 °C and kept at 500 °C for 2 h in air. Alpha minimum essential medium (α-MEM, Gibco/Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% antibiotics was used to culture osteoblastic cell line MC3T3-E1 (Riken Biobource Centre, Tsukuba, Japan) in a humidified atmosphere containing 5% CO₂ at 37 °C. Before seeding the cells, specimen pre-incubation with the culture medium for 24 h was prepared in a 48-well plate. After pre-incubation, removing pre-incubated medium and seeding cells on to surface of block specimens were progressed. For initial cell attachment, cells were cultured at a density of 1 × 10⁴ cells/well for 3 h. A Scanning Electron Microscope (SEM:
S-3400N, Hitachi High-Technologies Co., Tokyo, Japan) at 5 kV of accelerating voltage was used to observe the morphology of cells. The specimen was then taken out from the well and prepared for imaging by dehydration, fixation and gold sputter coating before taking images [17]. For the cell proliferation test, cells were seeded at a density of $1 \times 10^4$ cells/well and analyzed after 3 and 7 days using Presto Blue Assay (Biosource International, Camarillo, CA). At the 3rd and 7th days, 0.5 ml culture medium with 10% Prestoblue reagent was supplied after washing and discarding old medium. After incubation for 1 h, transferring 100 μl of the solution from each well to the 96-well-plate in triplicate was done to analyze.

| Specimen name | The loss of ignition (% wt) | Color       |
|---------------|-----------------------------|-------------|
| As-prepared powder | —                           | Light yellow|
| 300 °C        | 4.73                        | Grey        |
| 500 °C        | 6.20                        | White       |
| 700 °C        | 7.67                        | White       |
| 900 °C        | 9.15                        | White       |
Table 2. Lattice parameters of as-prepared powder and calcined powders calculated from XRD data.

| Specimen name                  | \(c (\text{Å})\) | \(a (\text{Å})\) | \(c/a\) |
|--------------------------------|------------------|------------------|---------|
| As-prepared powder             | 6.8852           | 9.1553           | 0.7520  |
| 300 °C                         | 6.8827           | 9.1372           | 0.7533  |
| 500 °C                         | 6.8825           | 9.1344           | 0.7535  |
| 700 °C                         | 6.8863           | 9.1182           | 0.7552  |
| 900 °C                         | 6.8665           | 9.0873           | 0.7536  |
| Standard HAp (ICDD PDF card #9432) | 6.8840           | 9.4180           | 0.7309  |

Results

Table 1 demonstrated the effect of calcination temperature on the color and the weight loss of the as-prepared powder calcined from 300 °C—900 °C. The results indicated that at calcination temperature of 300 °C, the color of the as-prepared powder changed from light yellow to grey. The as-prepared powder became completely white as calcined from 500 °C—900 °C and the loss on ignition of powder was also increased.

Figure 1 depicted the temperature-dependent mass change (TG), rate of mass change (DTG) and the heat flow (DSC) curves of the chicken bone measured in air. Below 900 °C, three mass loss steps of \(-1.87\%\), \(-5.76\%\) and \(-2.22\%\) were observed with maxima in the mass loss rate at 128 °C, 335 °C and 771.6 °C, respectively. Accompanying these mass loss steps, the DSC-signal exhibited peak at temperatures of 128.7 °C and 771.9 °C correlating very well with the above mentioned mass loss steps. The origins of the mass losses below 500°C were probably due to the release of residual humidity and due to the decomposition of the organic compounds. The detected DSC peak at 771.9 °C was most likely due to a decomposition of carbonate group.

The results of XRD analysis indicated that apatite was the only crystallite phase detected in the as-prepared and calcined powders (shown in figure 2). Apatite peaks of 300 °C and 500 °C for the calcined powder were similar with those of as-prepared powder. When increasing the heat treatment temperature to 700 °C and 900 °C, XRD peaks belonging to apatite phase became sharp and narrow. This implied that the crystallite size increased significantly during heat treatment above 500 °C. The Scherrer equation was used to estimate the crystallite size [18]. Peak (002) at 2\(\theta\) = 26° was selected for calculation since it was a typical apatite peak and it is not overlapped by the other peaks. At 300 °C and 500 °C, only physical water evaporation and organic residue burning occurred, therefore crystallite size was approximately 41.4 nm, this is almost the same with the as-prepared powder. Increasing heating temperature to 700 °C increased crystallite size to 69 nm. At 900 °C, crystallite size decreased to about 59 nm. One possible explanation for this is that from 900 °C CO3Ap started to be transformed to beta tricalcium phosphate (\(\beta\)TCP) [19] and that the phase transformation was just beginning, meaning that the \(\beta\)TCP phase was negligible and that it could not be detected in XRD pattern.

Table 2 summarizes the lattice parameters calculated from XRD data. The standard card of hydroxyapatite (ICDD PDF card #9432) indicated that standard hydroxyapatite has a hexagonal close packed structure with lattice parameters \(a = 9.418\) Å and \(c = 6.884\) Å. The \(c/a\) ratios of unit cells belonging to as-prepared powder and 300 °C–900 °C calcined powders showed an increase as compared with the \(c/a\) ratio of standard hydroxyapatite (as shown in table 2).

Lattice parameters of as-prepared and calcined powders showed a decrease in the \(a\)-axis as compared with the \(a\)-axis of standard hydroxyapatite (ICDD PDF card #9432). In the \(c\)-axis, the lattice constants either increased for as-prepared powder and powder calcined at 700 °C or decreased for powders calcined at 300 °C, 500 °C, and 900 °C as compared with the \(c\)-axis of standard hydroxyapatite (ICDD PDF card #9432). According to LeGeros RZ et al. in 1969, there are two types of carbonate substitutions in the apatite crystals. A-type CO3Ap in which CO3 groups replace OH groups can be obtained and stable at high temperature [20]. Crystal structure of A-type has an expanded \(a\)-axis and contracted \(c\)-axis. In B-type CO3Ap, the smaller (CO3)2− groups replace the larger (PO4)3− tetrahedra groups causing the \(c\)-axis to expand and the \(a\)-axis to contract. B-type CO3Ap obtained at low temperature is similar in structure to bone apatite and has low crystallinity. The as-prepared powder was similar to B-type lattice parameter since it was extracted from chicken bone without subsequent heat treatment. At low temperature heat treatments from 300 °C–500 °C, the contraction of both \(a\) and \(c\) axis could imply the presence of both A and B types. Although, the powder calcined at 700 °C had the lattice parameters similar to those of B-type, its crystal size was larger than that of the as-prepared powder. In that case, structural behavior of its unit cell could be due to the thermal stresses. At 900 °C, the changes in crystal structure and unit cell size distortion could be a result of phase transformation from apatite to \(\beta\)TCP during heat treatment.
Figure 3 showed the FTIR spectra of as-prepared powder, 300 °C, 500 °C, 700 °C, 900 °C calcined powder and reference hydroxyapatite (CAS. NO. 1306-06-5, Taihei Chemical Industrial Co., Ltd. Osaka, Japan).

Figure 4. The difference of absorption peak intensities at 875 cm⁻¹ of the as-prepared and calcined powders.

Figure 3 showed the FTIR spectra of as-prepared powder, 300 °C, 500 °C, 700 °C, 900 °C calcined powder and reference hydroxyapatite revealing typical bands for the carbonate apatite structure. Reference Hydroxyapatite (HAp) is the commercial synthetic HAp with high crystallinity and with no carbonate group in its structure. FTIR spectrum of reference HAp indicated peaks of PO₄ groups at 1100–980 cm⁻¹, 960 cm⁻¹ and 600–560 cm⁻¹ together with peak of OH groups at 630 cm⁻¹ [21]. The as-prepared and calcined chicken bone powder also had typical PO₄ groups belonging to apatite phase as shown in their FTIR spectra. A major characteristic of the as-prepared and calcined chicken bone powder in the present study was the presence of the
doublet bands of carbonate groups observed at 1450 cm\(^{-1}\) – 1420 cm\(^{-1}\) and singlet band at 875 cm\(^{-1}\). This was due to the substitution of phosphate groups (PO\(_4^{3-}\)) by carbonate (CO\(_3^{2-}\)) groups [22, 23]. It could imply that CO\(_3\)Ap obtained from chicken bone was B-type CO\(_3\)Ap usually present in biological apatite or natural bone.

Figure 4. indicated the difference of absorption peak intensities at 875 cm\(^{-1}\) of the as-prepared and calcined chicken bone powders. The singlet band at 875 cm\(^{-1}\) is the typical peak of carbonate group belonging to CO\(_3\)Ap. As shown in figure 4, as-prepared powder had the most intense band compared to calcined powders. As the heat-treatment temperature increased, this absorption peak decreased in intensity. Low intensity usually means a decrease in the amount of carbonate content in the apatite structure. The major decrease in intensity presented in powder heated above 500°C. That means above 500°C carbonate group was decomposed significantly and this was also in agreement with TG-DSC result.

Heat treatment is useful for removing organic residues completely, however it would cause the loss of carbonate content. The carbonate groups in apatite structure are the important factor to enhance bioactivity and cell response. Therefore, heat treatment temperature could be selected the temperature at which debris could be fully eliminated and carbonate groups were still in the range of 4–8 mass% mimicking carbonate content of natural bone apatite [1, 2]. For that reason, heating at 500°C would be suitable for heat treatment. Table 3 indicated the carbonate content of as-prepared powder and 500°C calcined powder measured by CHN analysis. Although the carbonate content of 500°C calcined powder showed approximately 62% decrease compared with that of as-prepared powder (p < 0.05), it was still in the range of natural bone apatite and, therefore, it confirmed that 500°C was the prospective selection of heat treatment processing for producing bone substitutes.

Figure 5 illustrated the morphology and particle size of the as-prepared powder and the 500°C calcined powder. The particle size and morphology were evaluated using transmission electron microscopy (TEM). As shown in figure 5, particles of as-prepared powder were in polygonal and rod shapes. The diameters of polygonal

![Figure 5](image)

Figure 5. TEM images of as-prepared powder (a)–(c) and 500°C calcined powder (e)–(g) at different magnifications.

| Sample name | Carbonate content (mass%) |
|-------------|--------------------------|
| As-prepared powder | 14.1 ± 0.4 |
| 500°C | 5.3 ± 0.1 (p < 0.05) |
particles were approximately 32 ± 5 nm. The rod-like particle were about 55 ± 12 nm in length and 10 ± 2 nm in diameter. Particles of 500 °C calcined powder only appeared in polygonal shape with diameters about 64 ± 16 nm.

Difference of particle shapes could be attributed to the presence of some organic residues remaining in the as-prepared particles. When powders underwent heat treatment, organic debris was burned completely and the coarsening of the particles also occurred.

Figure 6. The initial cell attachment after 3 h cultured on the surface of 500 °C calcined specimen and the surface of plastic well plate without specimen as the control sample.

Figure 7. SEM images showing the morphology and distribution of the osteoblastic MC3T3-E1 cells after 3 h cultured on the surface of 500 °C calcined specimen. The images capture two different areas (a) and (c) with (b) and (d) are the magnified images, respectively. The arrows indicate locations of the cells.
The *in vitro* evaluation using the osteoblastic MC3T3-E1 cells was conducted to check the cell response to the 500 °C calcined specimen. Figure 6 demonstrates the initial cell attachment after 3 h cultured on the surface of 500 °C calcined specimen and on the surface of plastic well plate without specimen as the control sample. After 3 h of incubation, 75% of cell population attached successfully to the surface of 500 °C calcined specimen.

Figure 7 showed SEM images of the morphology and distribution of the osteoblastic MC3T3-E1 cells after 3 h cultured on the surface of 500 °C calcined specimen. It could be seen that cells exhibited polygonal shape with filopodia and lamellipodia leading edge. Cell spread on the surface during migration and proliferation. Cell viability and spreading is one of the most important indicators for biocompatibility evaluation of material.

Figure 8 indicated the proliferation of the osteoblastic MC3T3-E1 cells on the surface of 500 °C calcined specimen and on the surface of plastic well plate without specimen as the control sample after 3 and 7 days of incubation. In this study, Presto Blue Assay was the method for determining cell proliferation based on metabolism. The number of cells increased very well with prolonged time of culturing. Since the cells presented a good proliferation with time, it proved that the material had high biocompatibility and no cytotoxicity as well as it could support cell biological activities. Hence, it implied that 500 °C calcined powder derived from chicken bone could be ready to be used for fabrication of bone substitutes.

**Discussion**

It was proved that evaporation of water as well as burning of organic matters occurred effectively with increasing heat treatment temperature since the weight of powder decreased and the loss on ignition increased. However, the losses on ignition of chemically pretreated powders in this study were less than those of untreated powders at the same temperatures [24]. This implied that pre-chemical treatment was effective to significantly remove the organic residues; hence, the subsequent heat treatment temperature could decrease to obtain required properties for clinical application. According to Shipman et al (1984)'s study, the apatite crystallite size did vary with increasing calcination temperature [25]. Bioactivity is one of the important properties for material in clinical application and small crystallite size with high surface area are found in materials of good bioactivity. Vallet-Regi et al (2004) reported that low crystallinity and inner crystalline disorder due to the presence of carbonate ions in the crystal lattice were two distinct features of CO₃Ap leading to its special bioresorbability [1]. Bioresorbability depends on the process of osteoclast activation on material. During bone remodeling process, osteoclasts form the Howship’s lacuna release proteolytic enzymes, cathepsin K, matrix metalloproteinase and hydrochloric acid. The Howship’s lacuna has low pH and is considered as acid environment due to the release of hydrogen ion by osteoclasts leading to the dissolution of bone minerals [26]. LeGeros RZ and Tung MS reported...
that higher carbonate content in apatitic structure was accompanied with increasing the solubility of apatite under weak acidic condition [27]. This means that carbonate content plays an important role in the cell-mediated bone resorption. Therefore, dissolution in the Howship’s lacuna region is limited in the case of high crystallinity hydroxyapatite (HAp: Ca_{10}(PO_{4})_{6}(OH)_{2}) which is free from carbonate ion in the structure. As a result, resorption of HAp by osteoclast is very low [3, 28–30]. Osteoclast resorption and osteoblast new bone formation are the parallel processes. Hence, good biodegradability of CO\textsubscript{3}Ap is favorable in osteogenesis because dissolution of CO\textsubscript{3}Ap can provide calcium and phosphate rich environment needed for bone formation [31–34]. For the CO\textsubscript{3}Ap derived from chicken bone, the subsequent heat treatment is necessary to eliminate completely organic residues however high temperature would lead to the high crystallinity and loss of carbonate groups. Therefore, suitable heat treatment needs to remove debris fully but also to maintain the carbonate content in the range of bone apatite. Based on the results, 500 °C was the prospective selection of heat treatment processing since it had a negligible effect on the crystal size and carbonate group. The in vitro evaluation using the osteoblastic MC\textsubscript{3}T\textsubscript{3}-E1 cells supported to clarify the cell-material interactions. Cell attachment on surface is one of the initial indicators of the upright cell-material interactions and the subsequent proliferation and differentiation are also determined by the degree of cell attachment. As mentioned above, the microstructure and composition of CO\textsubscript{3}Ap formed at low temperature resulted in its superior osteogenic properties. Cell activities are promoted strongly on surface that has bioactive chemistry and lower crystallinity topography [35, 36]. Accordingly, releasing of calcium could be superior with low crystallinity material. This is due to its active surface change creating energetic regions as well as accelerating the dissolution and precipitation reactions. As a result, precipitation of new apatite layer occurred and migration of cells was promoted. The greater surface reactivity could attract more osteoblasts adhering to particle boundaries via many interactions with initial proteins leading to subsequent cell adhesion. Carbonate groups in the apatite structure also supported effective protein adsorption and finally good cell adhesion. One of the characteristics deciding good cell adhesion is surface wettability. The hydrophilic surfaces tend to attract cell migration in comparison with hydrophobic surfaces. Hence, CO\textsubscript{3}Ap materials with low crystallinity and carbonate content in apatite structure possess hydrophilic surfaces to attract cell migration, proliferation and differentiation that could lead to the best integration to host bone during implantation [37].

Conclusions

Finding multidisciplinary applications for food industry by-products could solve the problem of waste. Inspired by the concept of turning waste into wealth, it was determined that a by-product from food industry is useful for producing bone substitute materials. In this matter, formation of carbonate apatite (CO\textsubscript{3}Ap) from chicken bone was discussed in this study. In overall, it has been demonstrated that this natural bio-waste can be a promising alternative for chemical-based precursor to produce CO\textsubscript{3}Ap. CO\textsubscript{3}Ap, derived from chicken bone, has the composition mimicking the inorganic content of the natural bone composition. Increasing the temperature of subsequent heat treatment could lead to the increasing of crystal size, the coarsening of particles and the loss of carbonate groups in apatite structure. The optimum temperature of heat treatment was found to be 500 °C. Heat treatment at this temperature could remove organic residues completely and retain the carbonate content in the range of bone apatite. The in vitro test results proved that CO\textsubscript{3}Ap derived from chicken bone and calcined at 500 °C had a good biological response including good cell attachment, cell viability and cell morphology.

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