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Ceramics International 33 (2007) 1171–1177
CERAMICS INTERNATIONAL
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Properties of hydroxyapatite produced by annealing of bovine bone

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Received 30 January 2006; received in revised form 26 February 2006; accepted 10 April 2006
Available online 14 August 2006

Abstract

The properties of porous hydroxyapatite (HA) bioceramic produced by heat treatment (annealing) of bovine bone were evaluated over temperatures between 400 °C and 1200 °C. The annealed body was characterized by thermal analysis (thermogravimetric analysis (TG)/differential thermal analysis (DTA)), scanning electron microscopy (SEM), X-ray diffraction (XRD), energy dispersive X-ray spectroscopy (EDX) and Fourier transformed infrared (FTIR) spectroscopy. The XRD results showed that the annealing process enhanced the crystallinity of HA phase in the bone matrix particularly when annealed above 700 °C. There was no secondary phase formation in bones annealed between 600 °C and 1000 °C. However, decomposition of HA to β-tricalcium phosphate (TCP) was observed for samples heat-treated at 1100 °C and 1200 °C. The FTIR spectra and the TG/DTA thermogram of as-received bovine bone indicated the presence of organic compounds, which upon annealing at temperatures above 600 °C was completely removed from the matrices. Bovine bone annealed between 800 °C and 1000 °C revealed the characteristics of a natural bone with the interconnecting pore network being retained in the structure.

Keywords: Bioceramics; Bovine bone; Hydroxyapatite; Annealing

1. Introduction

Operative treatment to replace bone that is lost due to accident/injury and/or bone defects resulting from inflammatory or chronic diseases has remained a challenge for orthopedic surgeons. Due to the limited supply of natural bone for grafting, the need for bone substitutes which have the same physicochemical and biological properties as natural bone is ever increasing. Although autogenous bone is most preferred for the treatment of bone defects, there are disadvantages and risks involved in using autogeneic bone such as post-operation pain, increased blood loss, secondary surgical wounds and risk of thrombosis [1–4]. Additionally, only a limited quantity of autogenous bone graft is available for harvest from a patient at any one time and this can be insufficient for children as well as adults requiring revision surgery.

Allograft bone could overcome the above limitations, but it bears the risk of transmission of infection (e.g. HIV, Hepatitis, etc.) [5,6]. Despite all of this, and amid concerns about its safety, the use of allogenous bone graft for skeletal restoration has been generally accepted and is likely to continue until alternative methods are found. Another possible alternative for treatment of bone defects is the use of xenogenous bone, which is morphologically and structurally similar to human bone.

Xenogenic bone is usually of bovine origin and is easy to obtain, lower cost and available in unlimited supply. At the material level, bovine bone is composed of organic and inorganic components. The organic part contains mainly collagen and proteins, whereas the inorganic component is mainly hydroxyapatite (HA) with a small percentage of other elements being incorporated in the structure such as carbonate, magnesium and sodium [7].

Recently, heat treatment has been suggested as an alternative to obtain protein-free bovine bone [8]. The crystalline phase composition of sintered bovine bone is similar to natural bone mineral which is composed of $Ca_{10}(PO_4)_6(OH)_2$ (i.e. HA) at about 93 wt% and about 7 wt% of β-tricalcium phosphate ($Ca_3(PO_4)_2$, β-TCP) [9]. The heat-treated bovine bone has an inter-connective porous structure (up to about 70 vol% porosity) and hence allows faster bone in-growth [8].

As with HA obtained from bovine bone, hydroxyapatite derived from powder processing route has great potential for

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0272-8842/$32.00
doi:10.1016/j.ceramint.2006.04.001

bone substitute owing to its excellent biocompatible and osteoconductive properties [9–11]. Moreover, using synthetic HA as bone substitute is advantageous, since it is non-inflammatory and causes no immunological or irritating response [12]. According to He et al. [13], HA can bond directly to tissues and promote tissue growth, which makes it the material of choice in orthopedic and dental applications. However, a major drawback of synthetic HA is its poor mechanical properties especially when exposed in wet environments [14–16]. Therefore, their clinical applications has been limited to low-load bearing applications such as tooth root substitutes, filling of periodontal pockets, cystic cavities, regions adjacent to implants, spinal fusions, contour and malformation defects and nonunions of long bones [17,18].

The most important parameters that can affect the properties of HA are the temperature and duration of heat treatment [19]. Zhou et al. [20] reported that synthetic HA with a Ca/P ratio near to 1.67 was stable below 1200 °C when sintered in a dry or moist atmosphere. HA suffers poor thermal stability and will decompose to form secondary phases such as tricalcium phosphate (TCP) when heat-treated at elevated temperatures above 1200 °C [21–23]. Hence, the influence of heat treatment on bovine bone at low and high temperatures is of great importance so as not to disrupt the HA phase stability.

The present study aimed at preparing HA directly from bovine bone through heat treatment (annealing) for use as a potential bone substitute. Beside that, this study also attempted to determine the chemical and physical properties of the resulting HA when annealed at different temperatures.

2. Experimental procedures

2.1. Sample preparation

A femur (plexiform bone) of an adult bovine (~2–3 years old) was procured from local slaughterhouse and cleaned to remove visible tissues and substances on the bone surface. It was then cut into rectangular samples of approximate size 10 mm × 5 mm × 5 mm. The as-received bone samples were annealed in an electric furnace (Elite Thermal System Ltd., UK), under ambient condition, at nine different temperatures ranging from 400 °C to 1200 °C, using a heating/cooling rate of 5 °C/min with 2 h holding time.

2.2. Characterization

The thermal transformation process of bovine bone into calcium phosphate ceramics and the hydroxyapatite thermal stability were studied by using a TG/DTA analyzer (Mettler Toledo 851^{e}, Switzerland). The thermogravimetric analysis (TG) and differential thermal analysis (DTA) of the bone samples during heating were recorded from 30 °C to 1000 °C at a heating rate of 10 °C/min in a stream of nitrogen (50 cm^3/min).

Phase analysis by X-ray diffraction (XRD) (Siemens® D5000 X-ray Diffractometer, Germany) of annealed samples was carried out at room temperature using Cu Kα as the radiation source at a scan speed of 0.5°/min and a step scan of 0.02°. The crystalline phase compositions were identified with reference to standard JCPDS cards available in the system software. The calcium (Ca) to phosphorus (P) ratio of annealed samples was calculated from compounds containing both elements.

The effect of annealing temperature on the microstructure evolution of the samples was examined using a scanning electron microscope (SEM: Philips XL40). The samples were mounted on aluminium stubs and subsequently coated with Au/Pd using a sputter coater (Polarons SC 7610, Fision Instruments). Chemical analysis was also carried out using energy dispersive X-ray spectroscopy (EDX) at 30.0 kV.

Fourier transformed infrared (FTIR) spectroscopy (Perkin-Elmer Instruments, Spectrum RX1) was also performed in order to understand the phase changes upon annealing and to determine HA stoichiometry deviations, i.e. the presence of anions partially substituting PO_4^{3-} and/or OH^- groups. For this measurement, the transmission IR spectra were recorded using KBr pellets (1 mg sample/300 mg KBr) over the range of 400–4000 cm^{-1} with 1 cm^{-1} resolution averaging over 100 scans.

3. Results and discussion

3.1. General observation

A direct observation that was made upon annealing at different temperatures was the colour change of the sample and this is given in Table 1. The colour of as-prepared bovine bone was light yellow. Upon annealing at temperatures of 400 °C, 500 °C and 600 °C the colour of the bone samples changed to black, dark grey and light grey, respectively. However, when annealing was performed at temperatures ≥700 °C, the samples were white in colour, suggesting complete removal of organic substances. This series of colour change is believed to be associated with the burn out process of organic matrix (e.g. protein and collagen) in the bovine bone. The darker colours observed for bone samples annealed below 700 °C indicated incomplete removal of organic compositions.

Table 1
Effect of annealing treatment on the colour of annealed bovine bone

Sample no.	Temperature (°C)	Colour
1[a]	Room temperature	Yellow (−)
2	400	Black
3	500	Grey (+)
4	600	Grey (−)
5	700	White
6	800	White
7	900	White
8	1000	White
9	1100	White
10	1200	White

(+) dark and (−) light.
[a] As-received bone.

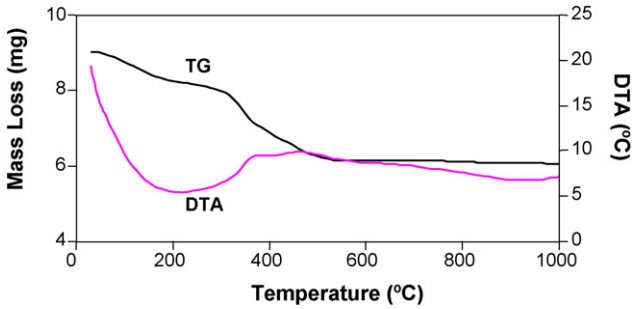

Fig. 1. Typical TG and DTA curve of as-received bovine bone from room temperature to 1000 °C.

3.2. Thermal analysis of bovine bone

The TG and DTA analysis of as-received bovine bone are shown in Fig. 1. From room temperature to about 200 °C, incorporated water is lost and this was supported by the DTA analysis, which showed a small endothermic transition at about 200 °C. A continuous weight loss was observed between 250 °C and about 550 °C, which can be associated with the burning of organic substances. There was no significant weight loss between 600 °C and 1000 °C, indicating that organic material such as fat tissues, collagen and proteins were completely removed at about 600–700 °C. Annealing above 600 °C, only the mineral phase (calcium phosphate) is left.

The average amount of water and organic phases removed during heat treatment was calculated and listed in Table 2. The results indicated that approximately 8.76% of water was lost when the bone sample was heated from room temperature to about 200 °C. A further approximately 24.45% of organics was removed between 250 °C and 550 °C. Therefore, it can be inferred that about 33% of total weight loss was due to removal of water and organic substances from the bovine bone when annealed up to about 600 °C.

3.3. XRD phase analysis

Fig. 2 presents the XRD patterns of as-received bovine bone and that annealed at varying temperatures. The XRD pattern of as-received bovine bone shows the presences of nanocrystalline apatite in the bone matrix. As the annealing temperature increased to 600 °C, the intensity of apatite characterized peaks gradually increased. The XRD signatures of bone annealed at 700 °C, 800 °C, 900 °C and 1000 °C were almost similar and exhibited a substantial increase in peak height and a decrease in peak width, thus indicating an increase in crystallinity and crystallite size. All the XRD signatures obtained for the bovine bone samples were in agreement with the stoichiometric HA characterized pattern (XRD JCPDS data file No. 9-432).

Table 2
Weight loss associated with heat treatment of bovine bone

	Weight (mg)		Weight loss (%)
	Before	After	
Amount of water removed (30–200 °C)	9.02	8.23	8.76
Amount of organic phase removed (250–550 °C)	8.14	6.15	24.45
Total			33.21

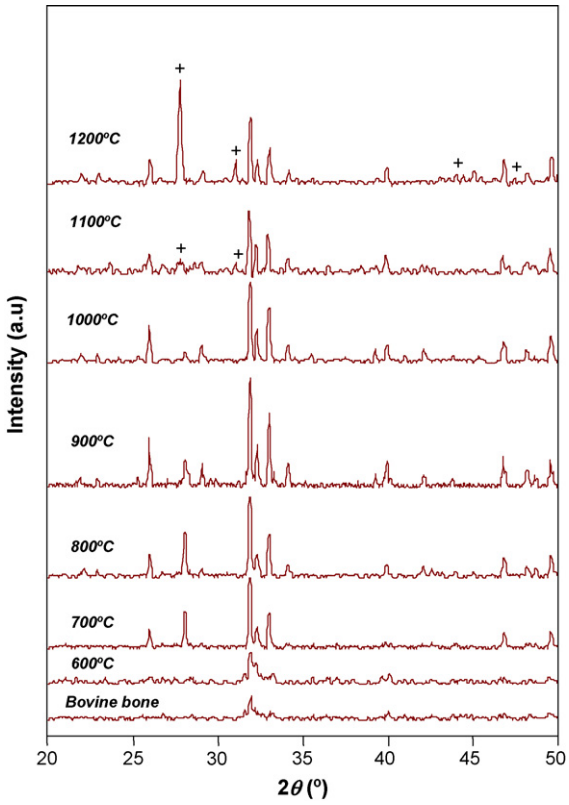

Fig. 2. X-ray diffraction patterns of as-received bovine bone and that annealed between 600 °C and 1200 °C (*key*: (+) β-TCP).

However, annealing above 1000 °C resulted in partial decomposition of the HA phase to form minor calcium phosphate phase of β-TCP (Fig. 2). As the annealing temperature increased to 1200 °C, the intensity of the characterized peaks for β-TCP, which is located at 2θ angles of 27.7°, 31.1°, 44.5° and 47.7°, was observed to gradually increase. The present XRD results suggest that the HA stability in the bone matrix was not disrupted when annealed in air up to 1000 °C.

It should be noted that although decomposition of HA phases was not detected in samples annealed up to 1000 °C, dehydroxylation of HA could have taken place. This can be observed by simply comparing the XRD peaks position of the annealed material with that of the standard JCPDS data for stiochiometric HA [24]. Table 3 presents the position of three most prominent XRD peaks position which corresponds to the plane (2 1 1), (3 0 0) and (2 0 2) of annealed bovine bone. In all cases, peak shifting was observed with 2θ values fluctuating between 0.004° (minimum) and 0.15° (maximum) from the standard position. This result indicates that dehydoxylation of the HA phase in bovine bone has occurred during annealing.

Table 3
Positions of XRD peaks of hydroxyapatite annealed between 700 °C and 1000 °C showing peak shifting due to dehydroxylation

	2θ (°) after annealing at various temperatures		
	(2 1 1)[a]	(3 0 0)[a]	(2 0 2)[a]
Standard	31.773	32.902	34.048
700 °C	31.901	32.912	34.044
800 °C	31.853	33.007	34.198
900 °C	31.887	33.023	34.139
1000 °C	31.810	32.953	34.146
Maximum difference	0.128	0.121	0.150
Minimum difference	0.037	0.010	0.004

[a] (*h k l*).

3.4. Microstructure evolution

Fig. 3(a and b) shows representative SEM pictures of as-received bovine bone and bones annealed at 400 °C, respectively. The microstructures of these samples appear to be dense due to the presence of organic substances in the bovine bone matrix. The morphology of bone annealed at 600–800 °C (not shown) exhibited a porous network remaining after the removal of organic materials from the bone. A typical bone-like matrix was observed for samples annealed ≥900 °C as shown in Fig. 3(c). From the surface morphology, the porous structure appears to be interconnected.

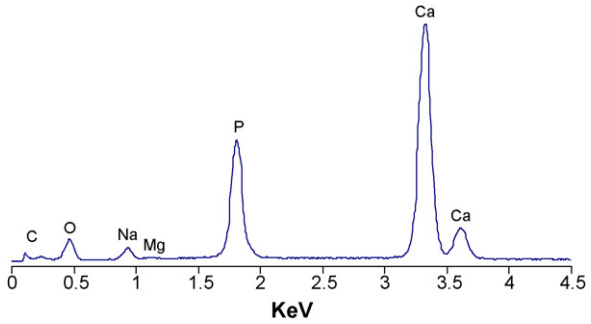

Fig. 4. EDX analysis of bovine bone annealed at 900 °C.

3.5. EDX analysis

A typical EDX spectrum showing the various elements that are present in the bone matrix is shown in Fig. 4. In general, the analysis indicated that the inorganic phases of annealed bovine bone composed mainly of Ca and P as the major constituents with some minor components comprising that of Na, Mg, O and C. The variation in Ca/P ratio of bovine bone annealed at various temperatures is shown in Fig. 5. In general, the Ca/P ratio of all samples was somewhat higher than the stoichiometric value of 1.67. The Ca/P ratio of as-received bovine bone was 2.23 and increased to 2.31 when it was annealed at 400 °C. Further annealing resulted in a decreased Ca/P ratio, down to about 1.85 for annealing at 1200 °C. Fig. 5 also shows that

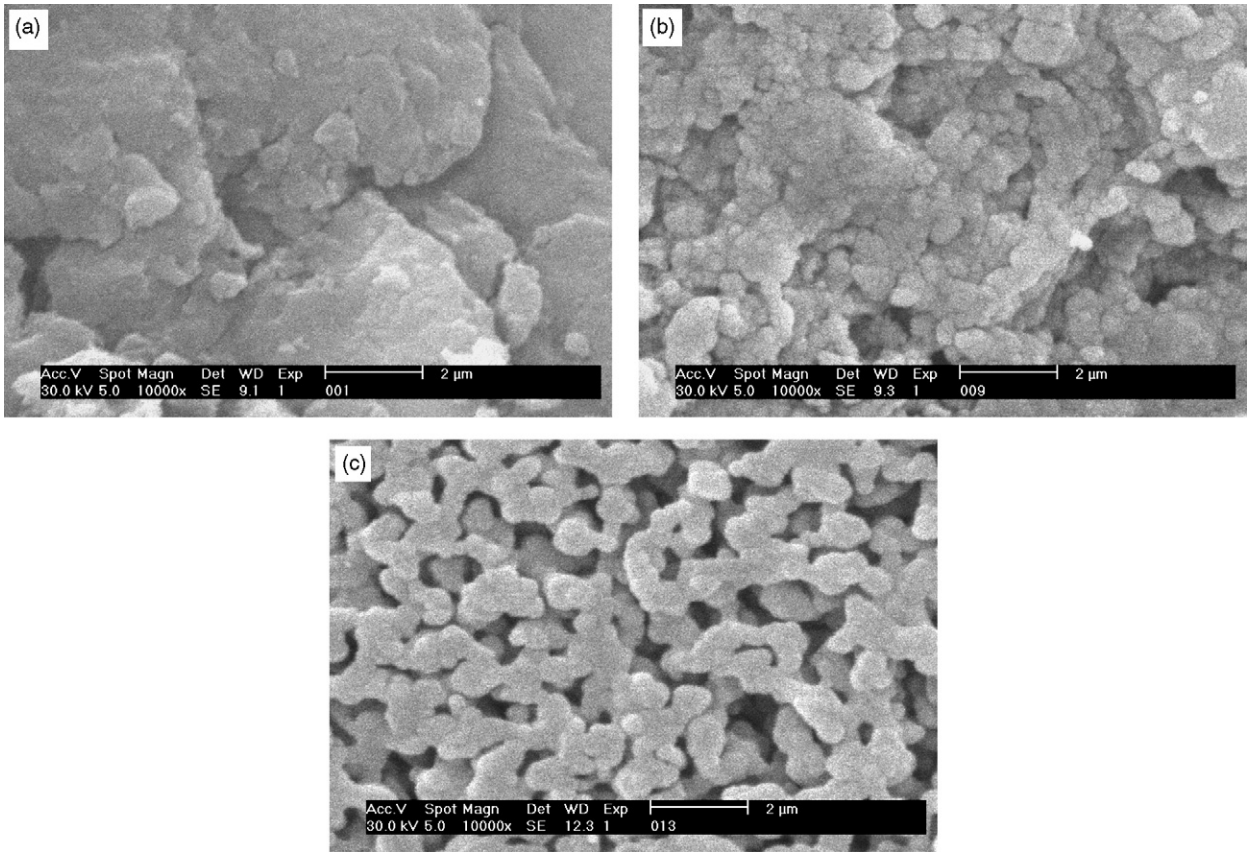

Fig. 3. SEM images of: (a) as-received bovine bone and, bone annealed at (b) 400 °C and (c) 900 °C.

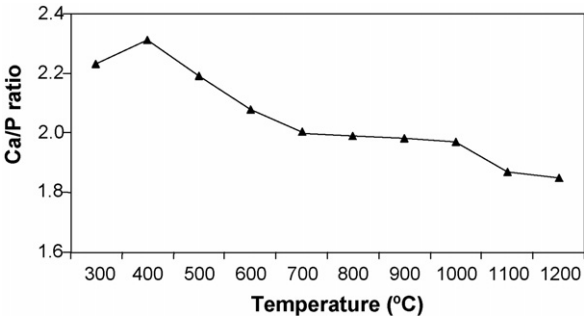

Fig. 5. Effect of annealing temperature on the Ca/P ratio of bovine bone.

when annealed at temperatures between 700 °C and 1000 °C, the Ca/P ratio did not changed significantly. It is unclear at this stage for the decline in the Ca/P ratio between 400 °C and 700 °C. Nevertheless, the drop in the Ca/P ratio for samples annealed at 1100 °C and 1200 °C may be associated with the partial decomposition of HA to form β-TCP as confirmed by XRD analysis. It should be noted, however, that the Ca/P ratio obtained for all samples in the present work is an approximate value and was intended to be used for comparison purpose only to evaluate the effect of increasing annealing temperature, as overlapping of peaks of different elements is possible but has not been investigated in detail.

3.6. FTIR analysis

The FTIR spectrum of as-received bovine bone and bone samples annealed at various temperatures are shown in Fig. 6. The IR spectrum of bone annealed between temperatures 700 °C and 1000 °C exhibited only the characteristic absorption peaks of HA. A large number of bands in the spectra (3571–3572 cm^{-1}, 3422.3–3425.1 cm^{-1}, 2072 cm^{-1}, 2002.5 cm^{-1}, 1411–1457 cm^{-1}, 1044–1049 cm^{-1}, 959–962 cm^{-1}, 631 cm^{-1}, 601.3–601.9 cm^{-1}, 568–571 cm^{-1}, 472.6–473.3 cm^{-1} and 425.5–426.1 cm^{-1}) matches the bands in the HA reference spectrum and are in close agreement with reported data on HA [19,25].

In the case of the as-received bovine bone, additional absorption peaks corresponding to a strong N–H stretching band around 2913 cm^{-1} and amide bands at 1544 cm^{-1} were observed. These two bands are characteristics of macromolecules of protein in the bovine bone matrix. They disappeared when the bone was annealed at 600 °C, thus indicating the complete removal of organic material and this is in good agreement with the observed colour change from black to grey and finally white. There are no significant differences observed in the FTIR spectra of bones annealed from 700 °C to 900 °C, suggesting no phase changes due to thermal effect.

In general, the FTIR spectra indicate the presence of phosphate (PO_4^{3-}), hydroxyl (OH^-) and carbonate (CO_3^{2-}) ions in annealed bovine bones. The 1049–1090 cm^{-1} bands arise from ν_3 PO_4^{3-}, the 962 cm^{-1} band arises from ν_1 PO_4^{3-} and the 560 cm^{-1} bands arise from ν_4 PO_4^{3-}. The group of weak intensity bands in the 1950–2200 cm^{-1} region derives from overtones and combinations of the ν_3 and ν_1 PO_4^{3-} modes

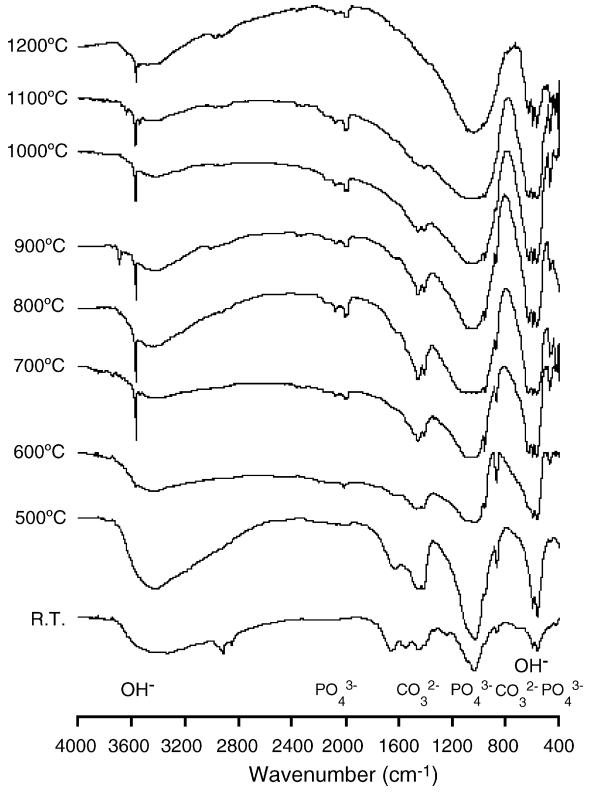

Fig. 6. FTIR spectra of bovine bone heated from room temperature (RT) to 1200 °C.

[26]. With increasing temperature from 500 °C to 1200 °C, the PO_4^{3-} bands of HA located at 560 cm^{-1}, 962 cm^{-1} and 1950–2200 cm^{-1} gradually increased. The weak bands at 1118 cm^{-1} and 945 cm^{-1} corresponding to TCP were also observed for samples annealed above 1000 °C.

Additionally, the FTIR spectra of all samples exhibited a pronounce peak at 630 cm^{-1} and 3570 cm^{-1} due to the presence of hydroxyl group. As the temperature is increased from 700 °C to 1000 °C, the band of OH^- at 3570 cm^{-1} gradually increases in its intensity with increasing temperature. This can be attributed to the increase in HA crystallinity with increasing annealing temperature as observed from the XRD patterns of these samples (Fig. 2). However, annealing above 1000 °C, the intensity band of OH^- at 3570 cm^{-1} started to decrease (Fig. 6). Such a behaviour is in accordance with the XRD analysis which indicated partial dehydration of hydroxyapatite, leading to decomposition of the HA phase for samples annealed at 1100 °C and 1200 °C (Fig. 2).

The intensity bands observed at about 1410 cm^{-1} and 1450 cm^{-1} in the spectrum of annealed bovine bones are attributed to components of the ν_3 mode of a trace amount of CO_3^{2-} and ν_2 CO_3^{2-} band at about 875 cm^{-1}. The FTIR spectra also showed the absorption peak of CO_3^{2-} ions around ν_2 and ν_3 modes in all samples except for bones annealed at 1100 °C and 1200 °C. The broad peaks from 1000 cm^{-1} to 1060 cm^{-1} can be explained owing to the C–O stretching vibrations.

In the present study, all the organic substances were eliminated by annealing in order to obtain antigenic free

inorganic bone minerals. The organic compositions were completely removed after annealing above 600 °C and this was confirmed by TG analysis, which showed no significant weight loss beyond this temperature regime. The weight loss (~33%) due to water and organic substances removal from the bovine bone is consistent with other studies [27,28], which indicated that bone is made up of about 70% of inorganic mineral crystals and about 30% correspond to the organic phases consisting of collagen protein fibers.

The DTA analysis in Fig. 1 shows the broad endothermic peak at about temperature 200 °C which corresponds to the great amount of water loss upon heating. However, the gradual decrease in weight from 600 °C to 1000 °C could be associated with dehydroxylation of HA and the slow elimination of the carbonate groups linked to bovine bone. This result is in agreement with FTIR analysis and a study by Landi et al. [29]. Based on the TG and DTA measurements in the present work and evidence provided in the literature [19], the weight loss can be explained by a series of processes: (1) the presence of adsorbed water that leaves the material, (2) organic materials such as collagen, fat tissue and proteins that burn out, (3) dissociation of carbonates or hydroxides to form oxides and (4) the start of dehydroxylation of the HA phase to form partial dehydrated oxyhydroxyapatite and oxyapatite, respectively.

From the XRD results obtained, the crystalline phase composition of annealed bovine bone is similar to that of hydroxyapatite when annealed at 700 °C, 800 °C, 900 °C and 1000 °C. TCP characterized peaks and other crystalline phases were not observed at these temperatures. According to Wang and Chaki [24], dehydroxylation of the HA phase would cause a small degree of peak shifting in the XRD trace. In the present work, it was found that the XRD 2θ position of bone samples annealed between 700 °C and 1000 °C shifted by 0.004–0.15°, thus indicating that the HA lattice had contracted due to loss of OH^- radicals.

It was also noted that the presence of small amount of β-TCP at 1100 °C and 1200 °C may be associated with the partial decomposition of HA phase. If HA is annealed in air at 1200 °C, it will decompose into the β-TCP phase according to equation [30,31]:

$$Ca_{10}(PO_4)_6(OH)_2 \rightarrow 3\beta\text{-}Ca_3(PO_4)_2 + CaO + H_2O_{gas} \quad (1)$$

The present XRD results are not consistent with reported literature which states that HA phase is stable in air up to 1200 °C [20,23]. This may be due to the present HA produced from bovine bone and not synthesized by a powder processing route. Based on the present result, it can be inferred that decomposition of HA produced from bovine bone takes place over 1000 °C so this temperature will be the highest temperature that can be used for the evaluation of HA properties of bovine bone. Since the conversion of HA into TCP results in the simultaneous formation of CaO and release of H_2O (Eq. (1)), changes in the activity of both CaO and H_2O should modify the location of the phase boundaries [32]. Since both dehydroxylation and decomposition reactions involve water vapour as a product, one can tailor the rates for these reactions by controlling the annealing atmosphere especially the water vapour partial pressure. One can also suppress the release of water vapour by encapsulating the HA grains or particles.

The SEM analysis of samples annealed >800 °C (Fig. 3) revealed a porous architecture that was created by the removal of organic phases. The annealed bovine sample maintained the spongy structure of natural bone, which has an interconnecting porous network that is suitable for bone in-growth to form an osseous bed.

The main difference between HA derived from a powder processing route and that obtained by annealing bovine bone is the absence of other minerals such as Na, Mg, K, F and Cl. From the EDX analysis as shown in Fig. 4, only Ca, P, Na and Mg were detected in the annealed bovine bone. This may be due to other minerals contents being too low to be detected by EDX. There are some factors that cause the Ca/P ratio for bovine bone to deviate from the theoretical Ca/P for pure HA. The annealing temperature and condition (with or without the presence of water pressure) will affect the type and amount of other calcium phosphate phase and/or other Ca compounds, which will be present with the HA phase [33]. The crystal lattice components of bovine HA such as Ca^{2+}, OH^- and PO_4^{3-} can readily be exchanged by other ions. Therefore, it is obvious that the composition of the trace elements varies considerably in bone depending on nutrition and the turnover rate of the mineral [19]. Commercially available HA can be deficient in phosphorous or in calcium ($Ca/P \neq 1.67$) while still yielding an X-ray diffraction spectrum identical to that of stoichiometric HA [23,30]. Alternatively, powders can have the right Ca/P ratio but contain a mixture of TCP and CaO [34]. Although the Ca/P ratio in this study deviates from the theoretical Ca/P for HA, XRD and FTIR analysis proved that annealing method has successfully produced the HA matrix from bovine bone. The higher values of Ca/P ratio obtained in the present work are in good agreement with that reported in literature for bovine bone [19].

The results obtained from FTIR analysis shows that bovine bone has a thermal behaviour similar to that of stoichiometric HA, which gradually releases its OH^- ions at high temperature [9,35]. Additionally, a small reduction in the amount of CO_3^{2-} was observed for the bone annealed below 500 °C, which may be due to the presence of organic substances in the bone matrix [36]. As expected, the bone heated at temperatures 800 °C, 900 °C and 1000 °C had much reduced CO_3^{2-} content. This result is consistent with the TG/DTA analysis and is in agreement with the study of Gross et al. [37], who reported that CO_3^{2-} may be removed by heating above 850 °C.

4. Conclusions

The present study shows the preparation of protein-free inorganic minerals of bovine bone by annealing above 600 °C in air atmosphere. Bovine bone annealed between 600 °C and 1000 °C exhibited a pure form of HA characterized patterns with no secondary phases present in the bone matrix. Additionally, the crystallinity of the HA phase in bovine bone increases with increasing annealing temperature. However, a

small amount of β-TCP was present in the bone matrix when annealed at 1100 °C and 1200 °C, resulting from partial decomposition of the HA phase. Nevertheless, the annealed (>700 °C) bone maintained a spongy structure of natural bone, which has an interconnecting porous network. The annealing method was found to be practicable in producing HA from bovine bone. Based on the results of this study, it is envisaged that with proper control of annealing temperature, HA produced from bovine bone has great potential to be used as a viable and economical graft material for clinical application.

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