Materials Science and Engineering C 31 (2011) 1136–1143

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Accelerated transformation of brushite to octacalcium phosphate in new biomineralization media between 36.5 °C and 80 °C

Neslihan Temizel, Giray Girisken, A. Cuneyt Tas *

Department of Biomedical Engineering, Yeditepe University, Istanbul 34755, Turkey

ARTICLE INFO

Article history:
Received 30 December 2010
Received in revised form 28 March 2011
Accepted 5 April 2011
Available online 12 April 2011

Keywords:
Calcium phosphate
Brushite
Transformation
Biomineralization

ABSTRACT

This study investigated the hydrothermal transformation of brushite (dicalcium phosphate dihydrate, DCPD, $CaHPO_4 \cdot 2H_2O$) into octacalcium phosphate (OCP, $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$) in seven different newly developed biomineralization media, all inspired from the commercial DMEM solutions, over the temperature range of 36.5 °C to 90 °C with aging times varying between 1 h and 6 days. DCPD powders used in this study were synthesized in our laboratory by using a wet-chemical technique. DCPD was found to transform into OCP in the Ca^{2+}, Mg^{2+}, Na^+, K^+, HCO_3^-, Cl^- and $H_2PO_4^-$ containing aqueous biomineralization media in less than 72 h at 36.5 °C, without stirring. The same medium was able to convert DCPD into OCP in about 2 h at 75–80 °C, again without a need for stirring. Samples were characterized by using powder X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM).

1. Introduction

We have recently shown that brushite (*dicalcium phosphate dihydrate*, DCPD, $CaHPO_4 \cdot 2H_2O$) powders soaked in commercial DMEM (*Dulbecco's Modified Eagle Medium*) solutions, at 36.5 °C for about one week, were able to completely transform into octacalcium phosphate (*octacalcium bis(hydrogenphosphate) tetraphosphate pentahydrate*, OCP, $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$) [1]. Brushite is a mildly acidic and highly soluble ($\log K_{SP} = -6.59$) calcium phosphate-based biomaterial which was only known to transform into apatitic calcium phosphate or hydroxyapatite (simplified by the popular formula of $Ca_{10}(PO_4)_6(OH)_2$, HA) when soaked in Tris (*tris-hydroxymethyl-aminomethane*)-buffered synthetic body fluid (SBF) solutions [2–4]. DMEM solutions are among the most preferred cell culture growth media and they contain amino acids, vitamins, inorganic salts, glucose and Hepes (*2-(4-(2-hydroxyethyl)-1-piperazinyl)ethane sulphonic acid*). While the Tris-buffered SBF solutions (with their HCO_3^- concentration made equal to that of the human blood plasma, i.e., 27 mM [5]) have a Ca/P molar ratio of 2.5, commercial DMEM solutions had the same ratio equal to 1.99 and a HCO_3^- concentration of 44 mM [1].

Tang et al. [6] had measured the dissolution rates of brushite, OCP and HA in water at RT as 4.26×10^{-4}, 1.63×10^{-6}, and 1.5×10^{-7} mol m^{-2} min^{-1}, respectively. The facile hydrothermal conversion of brushite into a moderately soluble calcium phosphate phase, such as OCP (log $K_{SP} = -72.5$ [7,8]), other than the quite low solubility of hydroxyapatite (log $K_{SP} = -117.1$ [8]) attracts interest towards the development of more soluble and bioactive bone substitute biomaterials.

Implanted brushite causes inflammation in the surrounding tissues [9–11], owing to its acidity, but OCP certainly does not [12–17]. More importantly, OCP is the precursor of the bone mineral [18]. It should always be the clear choice for the orthopedic or dental surgeon not to dictate the bone defect he/she wants to fill and repair by using a man-made non-resorbable hydroxyapatite but to use the precursor of the bone mineral (e.g., OCP) and thus help the bony tissue to repair itself naturally by using that precursor scaffold.

It has recently been made possible to produce self-setting cements of high in vivo resorbability with the setting product being brushite [9–11] instead of non-resorbable hydroxyapatite. Such attempts to develop brushite cements opened up an avenue to produce porous or non-porous bulk samples of brushite, in stark contrast to the already known production of brushite powders.Moreover, brushite can also be coated on metallic implants by using either electrolytic [19–21] or electroless [22] coating processes. Such bulk samples of brushite once produced would then need the development of robust methods of transformation into OCP (instead of hydroxyapatite [23]) by either using perfectly biomimetic solutions (i.e., DMEM solutions used in cell culture) as we have shown in our previous study [1] or the biomineralization media developed in the current study.

The current study [24,25], as being a direct follow-up to our previous work on transforming brushite (DCPD) into OCP in DMEM solutions at 36.5 °C [1], is aimed at finding answers to the following questions;

(i) Are those amino acids, vitamins, glucose and Hepes present in the DMEM solutions really necessary for the transformation of DCPD into OCP?

* Corresponding author at: Current address: Department of Dental Materials, University of Oklahoma College of Dentistry, OUHSC, Oklahoma City, OK 73117, USA. Fax: +1 405 271 2092.
E-mail address: cuneyt-tas@ouhsc.edu (A.C. Tas).
URL: http://www.cuneyttas.com (A.C. Tas).

0928-4931/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.msec.2011.04.009

(ii) Are the Mg^{2+} and/or HCO_3^- ions present in DMEM solutions really necessary for the DCPD to OCP transformation?
(iii) Does the Ca/P molar ratio of those solutions influence the DCPD to OCP transformation?
(iv) Could it be possible to shorten the time of transformation of DCPD to OCP by increasing the temperature of the biomineralization media?

2. Materials and methods

2.1. Synthesis of brushite powders

Brushite crystals of this study were prepared by using two different, but quite similar chemical synthesis procedures. The first procedure used to synthesize flat plate-shaped brushite crystals simply consisted of preparing two solutions. Solution A was prepared as follows: 0.825 g of KH_2PO_4 (≥99.9%, Cat. No: 104873, Merck KGaA, Darmstadt, Germany) was dissolved in 700 mL of double-distilled water contained in a 1 liter-capacity glass beaker, followed by the addition of 3.013 g of Na_2HPO_4 (≥99.9%, Cat. No: 106586, Merck), which resulted in a transparent solution of pH 7.5 at room temperature (RT: 21 ± 1 °C). Solution B (of pH 6.4) was prepared by dissolving 4.014 g of $CaCl_2 \cdot 2H_2O$ in (≥99.9%, Cat. No: 102382, Merck) 200 mL of double-distilled water. Solution B was then rapidly added to solution A and the precipitates formed were aged for 80 min at RT, by continuous stirring at 500 rpm (final solution pH 5.3). Solids recovered by filtration (Whatman Filter paper, No. 1 or 2 or 4) from their mother liquors, followed by washing with 1.5 L of distilled water, were dried at 36.5 °C for 48 h to obtain approximately 3.3 g of brushite powders. These powders are designated as Na–K-DCPD.

The second procedure used to synthesize the brushite crystals only differed in the preparation of the Solution A, and the rest of the method was exactly identical with the one described above. Solution A of the second procedure was prepared as follows: 1.395 g of $NH_4H_2PO_4$ (≥99.9%, Cat. No: 101126, Merck) was dissolved in 700 mL of double-distilled water, followed by adding 6.501 g of $(NH_4)_2HPO_4$ (≥99.9%, Cat. No: 101207, Merck), which also gave a transparent solution of neutral pH at RT. These powders are designated as NH_4-DCPD.

These two powders were tested in every experiment of this study, i.e., if one experiment was performed with the first powder, the repeat experiment was intentionally performed with the second brushite powder. While the second type of brushite powders was synthesized in the presence of NH_4 ions, the first type was prepared in the absence of those. Therefore, this study was also planned to test the significance of ammonium ions (in synthesizing the starting material brushite) on the obtained results.

2.2. Preparation of biomineralization media

The newly developed biomineralization media were prepared as shown in Table 1. Chemicals are added, in the order shown in Table 1, to 1 L of double-distilled water contained in a clean glass media bottle, including a 4 cm-long Teflon®-coated magnetic stir bar. To clarify, the first chemical added was Ca-chloride dihydrate and the second chemical $MgCl_2 \cdot 6H_2O$ (>99.5%, Cat. No: 459330, Carlo Erba Reagenti, Milano, Italy) was not added before the complete dissolution of the preceding one, under vigorous stirring on a stir plate. Media were prepared at room temperature. KCl (>99.9%, Cat. No: 104933, Merck), $NaHCO_3$ (>99.9%, Cat. No: 106329, Merck), NaCl (>99.5%, Cat. No: 106404, Merck), $NaH_2PO_4 \cdot 2H_2O$ (>99.5%, Cat. No: 106342, Merck), Hepes ($C_8H_{18}N_2O_4S$, >99.9%, Cat. No: 110110, Merck) and gelatin (>99.5%, Cat. No: 104078, Merck) were also used in preparing these media.

Briefly, as shown in Table 1, BM-1 maintains the inorganic salts portion of the commercial DMEM solution [1], subtracts glucose, all vitamins and amino acids from it but brings in some amino acids by using soluble gelatin, while keeping Hepes. The only difference between BM-2 and BM-1 was the elimination of gelatin. In passing from BM-2 to BM-3, Hepes was eliminated. BM-4 media removed Mg-chloride from BM-3, BM-5 eliminated $NaHCO_3$ from BM-3, whereas BM-6 removed both Mg-chloride and $NaHCO_3$ from the BM-3 solutions. BM-7 increased the Ca/P molar ratio of BM-3 from 1.99 to 2.5. Finally, the only difference between BM-7 and BM-8 media was the addition of Hepes in the latter.

2.3. Transformation of brushite to OCP in the biomineralization media

Glass media bottles (100 mL-capacity) containing 100 mL of BM solutions of Table 1 were used. 650 mg of brushite powder was placed in each bottle and the plastic caps of the bottles were sealed. The bottles were placed in a microprocessor-controlled static air oven whose temperature was adjusted to either 36.5 (±0.1 °C), 50, 70, 75, 80 or 90 °C. The times for aging the brushite powders in the BM solutions at 36.5 °C were selected as 24, 48, 72 and 144 h. The BM solution of the 144 h samples was replenished with a fresh solution at every 48 h. The BM solution of the 72 h samples was replenished after the 48th hour. Only the BM-3 media was used for the experiments performed at temperatures higher than 36.5 °C. The experimental conditions for the high-temperature samples were given in Chapter 3. At the end of specified aging periods, the solids were recovered from the media by using a porcelain Buechner funnel and No. 2 Whatman filter paper, with vacuum filtration. The solids were washed with 700 mL of double-distilled water. Washed samples were dried at 36.5 °C for 48 h.

2.4. Sample characterization

All powder samples were characterized by using a powder X-ray diffractometer (Advance D8, Bruker AG, Karlsruhe, Germany) after grinding with an agate mortar and pestle. The diffractometer was operated with a Cu tube at 40 kV and 40 mA equipped with a monochromator. Samples were scanned with a step size of 0.02° and a preset time of 5 s.

Table 1
Preparation of biomineralization media used in the 36.5 °C experiments*.

	BM-1	BM-2	BM-3	BM-4	BM-5	BM-6	BM-7	BM-8
$CaCl_2 \cdot 2H_2O$	0.2646 (*1.80*)	0.2646	0.2646	0.2646	0.2646	0.2646	0.3323 (*2.26*)	0.3323 (*2.26*)
$MgCl_2 \cdot 6H_2O$	0.1655 (*0.81*)	0.1655	0.1655	–	0.1655	–	0.1655	0.1655
KCl	0.3974 (*5.33*)	0.3974	0.3974	0.3974	0.3974	0.3974	0.3974	0.3974
$NaHCO_3$	3.7005 (*44.05*)	3.7005	3.7005	3.7005	–	–	3.7005	3.7005
NaCl	4.7865 (*81.90*)	4.7865	4.7865	4.7865	4.7865	4.7865	4.7865	4.7865
$NaH_2PO_4 \cdot 2H_2O$	0.1413 (*0.90*)	0.1413	0.1413	0.1413	0.1413	0.1413	0.1413	0.1413
Hepes	5.9580	5.9580	–	–	–	–	–	5.9580
Gelatin	3.0000	–	–	–	–	–	–	–
Solution pH	7.40	7.45	7.40	7.40	6.70	6.65	7.40	7.40
		Gelatin-free	Hepes-free	Mg-free	CO_3-free	Mg & CO_3-free	Ca/P = 2.5	Ca/P = 2.5

* The amounts of chemicals needed were given in grams per liter of media, whereas the numbers in parentheses were the mM values of the cations. Ca/P molar ratio in media BM-1 through BM-6 was equal to 2.

Fourier-transform infrared spectroscopy (Spectrum One, Perkin Elmer, MA, USA) analyses were performed after mixing 1 mg of sample powders with 300 mg of KBr powder in an agate mortar, followed by compacting those into a thin pellet in a stainless steel die of 1 cm inner diameter. FTIR data were recorded over the range of 4000 to 400 cm^{-1} with 128 scans.

Scanning electron microscopy (EVO 40, Zeiss, Dresden, Germany) was used to evaluate the morphology of the powder samples. The samples were sputter-coated, prior to imaging, with a 25 nm-thick gold layer to impart electrical conductivity to the specimen surfaces.

3. Results

The XRD (x-ray diffraction), FTIR (Fourier-transform infrared spectroscopy) and SEM (scanning electron microscopy) characterization data of the starting powders of this study are combined into one chart and provided in Fig. 1. The brushite powders (both Na–K-DCPD and NH_4-DCPD type) were single-phase and of high purity. Brushite crystals were flat, optically transparent and plate-like with an average crystal length of 35 μm. The XRD reflection (at 2θ 11.68°) from the (020) planes of these crystals was very strong. The XRD pattern of these powders conformed well to the ICDD PDF (International Centre for Diffraction Data Powder Diffraction File) 9-0077.

Gelatin and Hepes-containing biomineralization solutions, BM-1, of Table 1 were not able to completely transform the DCPD powders into OCP in 24, 48 and 72 h of aging at 36.5 °C. However, the BM-1 solutions converted all the DCPD into OCP in 6 days at the human body temperature.

The behavior of gelatin-free but Hepes-containing BM-2 solutions was similar to those of BM-1, they were not able to fully convert the DCPD powders into OCP when the soaking time was less than 6 days at 36.5 °C.

Hepes- and gelatin-free BM-3 solutions completely transformed the DCPD powders into OCP (perfect match with the ICDD PDF 26-1056 for OCP) in 72 h at 36.5 °C, but at 24 and 48 h the transformation was incomplete. Hepes-, gelatin- and Mg-free BM-4 solutions displayed a behavior similar to those of BM-3 solutions, i.e., 72 h was the minimum time of soaking for complete transformation. BM-3-72 h samples also exhibited around 30 wt.% loss from the starting weight of DCPD to the final weight of OCP powders obtained. The results obtained with BM-1 through BM-4 were summarized in the XRD plots of Fig. 2a and the FTIR charts of Fig. 2b. In Fig. 2a, we have intentionally clipped the intense (020) reflection of BM-1-72 h sample's XRD trace not to let it interfere and overlap with the same reflection of the BM-2-72 h sample. The FTIR data were able to spot the characteristic bands of DCPD especially when it is in a biphasic mixture with OCP, as exemplified in the bottom trace of Fig. 2b.

The media BM-5 and BM-6 (of Table 1) tested the influence of the absence of HCO_3^- and $HCO_3^- + Mg^{2+}$ ions on the transformation behavior of DCPD to OCP at 36.5 °C in 72 h, respectively. As shown in the XRD data of Fig. 2c, the absence of HCO_3^- from the media had a strong effect and BM-5 and BM-6 media did not convert the DCPD powders into OCP in 72 h at 36.5 °C. The very intense (020) reflection of the unreacted DCPD phase observed in both traces was intentionally clipped to facilitate the better resolution of the lower intensity peaks present in the rest of the patterns.

The media BM-7 and BM-8 examined the role of increased Ca/P molar ratio (from 1.99 to 2.50) in the absence and presence of Hepes, respectively, in 72 h of aging at the human body temperature of 36.5 °C. The XRD data given in Fig. 2d showed that, in direct comparison to the sample of BM-3-72 h, both of BM-7 and BM-8 were able to fully convert the DCPD powders into OCP in less than 72 h.

The starting pH values of all the media (without the DCPD crystals) of Table 1 were over the narrow range of 7.4 to 7.5 at the time of media preparation, and the pH values were dropped to the range of 6.5 to 7 at the end of aging at 36.5 °C between 24 to 144 h, with the only exceptions of BM-5 and BM-6. This seemed to be a small but very consistent and meaningful pH decrease. Hepes present in especially BM-2 and BM-8 media was not able to prevent this pH drop in the presence of mildly acidic DCPD. For carbonate ion-free BM-5 and BM-6 media the initial pH values were around 6.7 and dropped to about 6.4 after 72 h of aging at 36.5 °C with the DCPD powders.

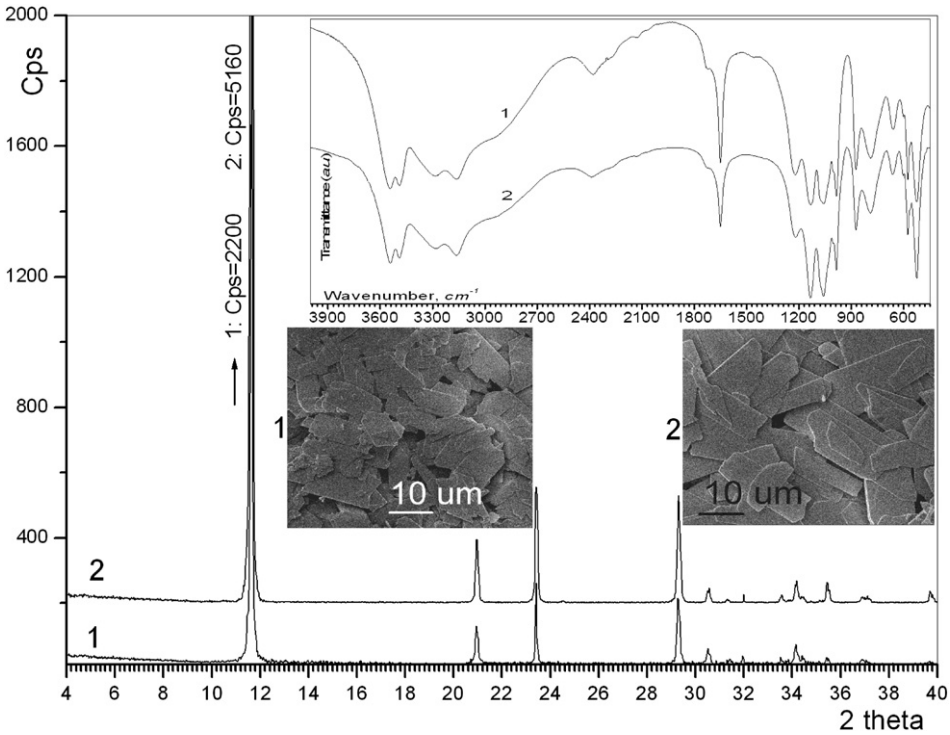

Fig. 1. Combined XRD, FTIR and SEM of brushite powders (*Sample 1: Na–K-DCPD and Sample 2: NH_4-DCPD*).

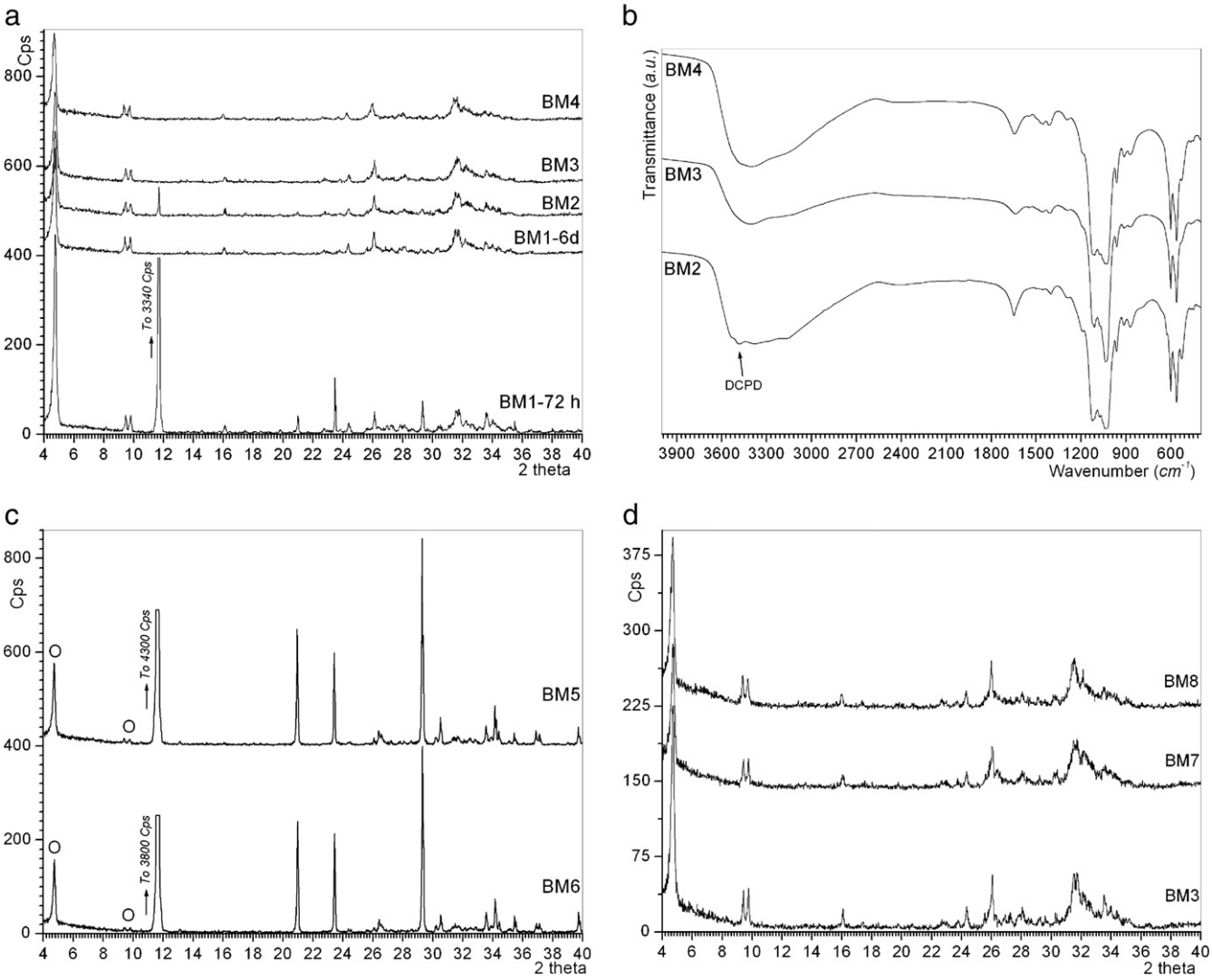

Fig. 2. a. XRD traces of Na–K-DCPD samples aged in BM-1 through BM-4 media at 36.5 °C for 72 h, *except otherwise noted.* b. FTIR traces of Na–K-DCPD samples aged in BM-2 through BM-4 media at 36.5 °C for 72 h. c. XRD traces of Na–K-DCPD samples aged in BM-5 and BM-6 media at 36.5 °C for 72 h. d. XRD traces of Na–K-DCPD samples aged in BM-3, BM-7 and BM-8 media at 36.5 °C for 72 h.

The effect of temperature in accelerating the hydrothermal transformation rate of the DCPD crystals into OCP was studied in both stirred and non-stirred aging media. For this study, the BM-3 media was selected and all the experiments of temperature-time study were performed with this solution (see Table 2) and by using both of Na–K-DCPD and NH_4-DCPD powders. The phase(s) observed after each experiment was also reported in Table 2.

Fig. 3a and b respectively showed the resultant XRD and FTIR data of 650 mg of DCPD samples stirred for 1 h in 100 mL of BM-3 solution in glass media bottles over the temperature range of 55 through 85 °C. None of these experiments were able to produce single-phase OCP. 55 °C–1 h was too low a temperature-time combination to achieve the DCPD to OCP transformation, whereas higher temperatures (60, 70 and 85 °C) favored the Ap-CaP (apatitic calcium phosphate) formation. It must be noted that increasing the temperature from 55 to 60 °C (for 1 h of stirring) was sufficient to fully decompose DCPD (Fig. 3a).

The XRD and FTIR data given in Fig. 4a–c summarized the results of high-temperature (50, 70, 75 and 80 °C) non-stirred experiments performed with the BM-3 solution. Keeping the DCPD powders in the BM-3 solution at 50 °C for 19 h was sufficient to transform them into OCP, this was a significant decrease achieved in aging time as compared to the 36.5 °C experiments (Fig. 4a). However, aging the DCPD powders at 70 °C in BM-3 for 1 or 2 h was not enough for the complete transition to OCP to take place (Fig. 4a–c). Soaking at 75 °C for 1 h was also not sufficient to eliminate all the DCPD (Table 2). The changes observed in the XRD

Table 2
Experimental parameters of the temperature-time study with BM-3.

Sample	Temp (°C)	Time (h)	Stirred*	pH	Phase(*s*)
HT-1	55	1	Y	6.8	DCPD + OCP
HT-2	60	1	Y	7.1	OCP + Ap-CaP
HT-3	70	1	Y	7.4	Ap-CaP
HT-4	85	1	Y	7.4	Ap-CaP
HT-5	50	19	N	6.8	OCP
HT-6	70	1	N	6.8	DCPD
HT-7	70	2	N	6.7	DCPD + OCP
HT-8	75	1	N	6.6	DCPD
HT-9	75	2	N	6.5	OCP
HT-10	75	2.33	N	6.5	OCP
HT-11	75	3	N	6.6	OCP
HT-12	80	1	N	6.8	OCP
HT-13	80	2	N	6.7	OCP
HT-14	90	4	N	6.9	OCP + Ap-CaP

* Stirring rate was kept constant at 265 rpm (whenever applicable).

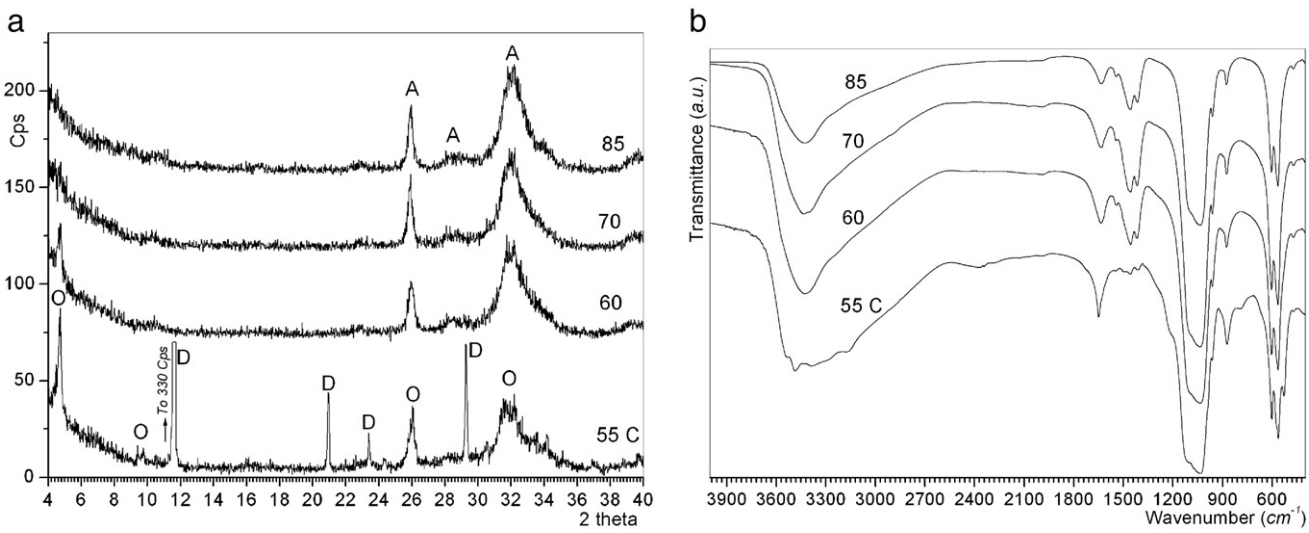

Fig. 3. a. XRD traces of NH_4-DCPD samples stirred in BM-3 media for 1 h at 55 °C, 60 °C, 70 °C and 85 °C (*O: OCP, D: DCPD and A: Ap-CaP*). b. FTIR traces of NH_4-DCPD samples stirred in BM-3 media for 1 h at 55 °C, 60 °C, 70 °C and 85 °C.

intensity of the (040) reflection (located at 23.39° 2θ) of brushite phase (as seen in the data of Figs. 1, 2a and 4a) were due to the use of ammonium phosphate salts in synthesizing the brushite powders of those samples. When we used ammonium phosphate salts in brushite synthesis, the XRD intensities of such samples were conforming to those given by ICDD PDF (*International Centre for Diffraction Data, Powder Diffraction File*) 11-0293; however, when we did not use ammonium salts in brushite powder synthesis the relative peak intensities were matching those listed by ICDD PDF 09-0077. The changes in peak intensities are not deemed to be so important in powder x-ray diffraction, since they can also be caused by preferred orientation effects. The d-spacings of the above-mentioned brushite samples did not change as a function of the spectator ions (ammonium, sodium or potassium) present in the brushite synthesis solutions.

We have experimentally found that the DCPD powders needed to be immersed, without stirring, in the BM-3 solutions at 75 °C for 2, 2.33 or 3 h for obtaining single-phase OCP. Soaking the DCPD powders at 80 °C in the BM-3 solutions for either 1 or 2 h, with no stirring, was also producing single-phase OCP (Table 2 and Fig. 4b and c). Soaking the same powders in BM-3 media at 90 °C for 4 h, on the other hand, produced biphasic OCP + Ap-Cap. Fig. 5a and b depicted the SEM morphology of the samples of BM-3-6 days–36.5 °C and HT-10 of Table 2, respectively. Both of these samples were free of any residual brushite, but most particles were retaining the DCPD morphology.

Finally, all the results reported above did not change when we changed Na–K-DCPD powder, as the starting material, to NH_4-DCPD powder for all the biomineralization media, temperatures and times studied.

4. Discussion

4.1. DCPD to OCP transformation

The overall transformation of brushite to OCP can be visualized by the below reactions. Reaction (2) helps to explain the effect of Ca^{2+} ions present in the biomineralization media.

$$10CaHPO_4.2H_2O(s) \rightarrow Ca_8(HPO_4)_2(PO_4)_4.5H_2O(s) + 2Ca^{2+} + 4H_2PO_4^- + 15H_2O \quad (1)$$

$$6CaHPO_4.2H_2O(s) + 2Ca^{2+} \rightarrow Ca_8(HPO_4)_2(PO_4)_4.5H_2O(s) + 7H_2O + 4H^+ \quad (2)$$

It should be noted that the extent of any hydrothermal transformation of a DCPD-based substance into OCP immersed in an aqueous solution shall strongly depend on the overall dimensions or thickness of the samples. The current study used micron-size powders and if the samples were bulky and much larger, then the transformation times determined and reported in this study (for submicron-thick plate-like brushite crystals) may not be directly applicable to such larger sizes.

Reactions (1) and (2) cannot explain the observation of the carbonate bands in the IR spectra (especially over the range of 1410 to 1470 cm^{-1}) of the OCP samples shown in Fig. 2b and c, and those bands are most probably indicative of the presence of quite small amounts of cryptocrystalline Ap-CaP. To differentiate between OCP and Ap-CaP is actually not so difficult, and the comparative FTIR data of Fig. 6a indicate the characteristic OCP bands by the + signs. Moreover, the appearance of the main phosphate bands located over the range of 990 to 1160 cm^{-1} can be practically used to ascertain the presence of OCP. The OCP in Fig. 6a was the sample of HT-10 of Table 2, whereas the Ap-CaP trace belonged to that of the HT-4 of the same table. The exact locations of the IR bands of OCP in our samples were coinciding well with those reported previously by LeGeros et al. [26,27] and Markovic et al. [28]. The IR spectra and IR band locations of the as-prepared brushite powders (Fig. 1) of this study conformed to those reported by Xu et al. [29].

To the best of our knowledge, DCPD to OCP hydrothermal transformation was not studied before in synthetic DMEM-like solutions. However, Tung et al. [30] studied the DCPD to OCP conversion in water over the pH range of 6.2 to 10.5 (solution pH values being adjusted by additions of H_3PO_4 and/or KOH) and at temperatures of 25 °C and 37 °C. When the solution pH values were between 6.2 and 6.8, DCPD transformed to OCP, whereas at higher pH values apatite phase formed [30].

Perez et al. [31] studied the transformation of DCPD to OCP in K-phosphate solutions containing nitrate ions (originating from the use of $Ca(NO_3)_2 \cdot 4H_2O$ as the calcium source) at 37 °C and at pH values between 6.0 and 7.0. At pH values of 6.4 and 6.7, Perez et al. [31] reported the transformation of the seed DCPD crystals into OCP without any difficulty.

4.2. Biomineralization media development

The biomineralization media developed in the current study (Table 1), to their advantage, contained Na^+, Ca^{2+}, Mg^{2+}, K^+, Cl^-, and HCO_3^- ions at concentrations not so different from those of human blood plasma.

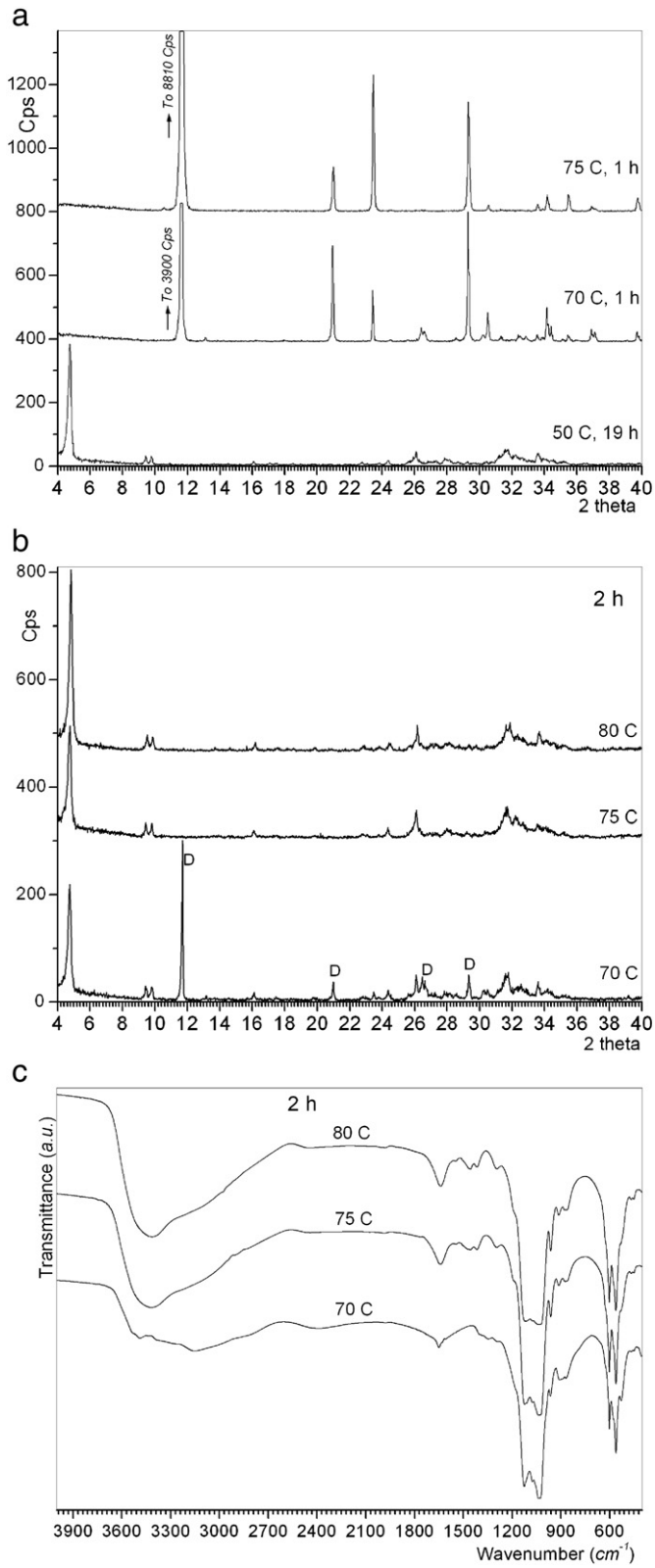

Fig. 4. a. XRD traces of NH_4-DCPD samples aged in BM-3 media for 19 h at 50 °C, 1 h at 70 °C, and 1 h at 75 °C. b. XRD traces of NH_4-DCPD samples aged in BM-3 media for 2 h at 70 °C, 75 °C and 80 °C. c. FTIR traces of NH_4-DCPD samples aged in BM-3 media for 2 h at 70 °C, 75 °C and 80 °C.

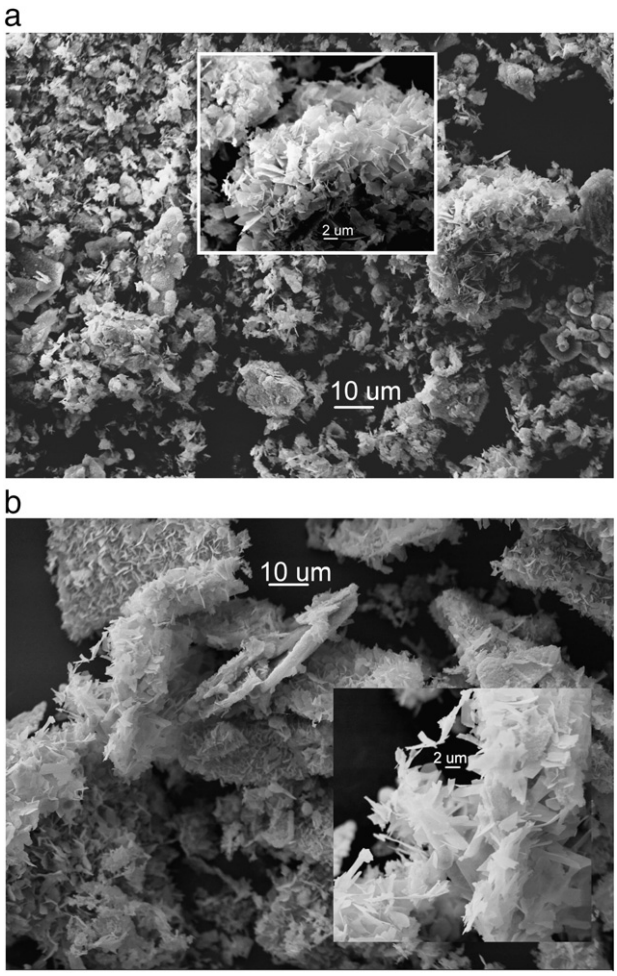

Fig. 5. a. SEM photomicrograph of Na–K-DCPD samples aged in BM-3 media at 36.5 °C for 72 h. b. SEM photomicrograph of NH_4-DCPD samples aged in BM-3 media at 75 °C for 2.33 h.

BM-1 solution was an attempt to prepare a synthetic solution free of the glucose and vitamins of a commercial DMEM solution, while maintaining the ionic constituents, Hepes and amino acids (as far as that can be achieved) of the DMEM at the proper concentrations. Eastoe [32] had reported the amino acid concentrations present in mammalian gelatin, and based on those concentrations we have estimated that the amount of gelatin given in Table 1 for BM-1 media could roughly approximate the amino acid concentrations of the commercial DMEM solution [1]. It is actually theoretically impossible to match the amino acid concentrations of the DMEM solution by using only gelatin. However, by using gelatin and Hepes we have actually obtained the same sluggish transformation kinetics for the brushite powders at 36.5 °C. BM-1 of the current study and the DMEM solution of our previous study [1] performed quite similarly and they both required about a week at the human body temperature for the DCPD to OCP conversion to occur (Fig. 2a). This is probably due to the adsorption of the amino acid molecules on the flat surfaces of the DCPD crystals, which would only hinder the hydrothermal transformation process [33,34]. When the gelatin was removed from the formula in preparing, for instance, the BM-2 media while keeping Hepes, the samples soaked in the BM-2 media for 72 h (at 36.5 °C) exhibited a much faster transformation rate in comparison to BM-1 (Fig. 2a). This proved that the amino acids originating from gelatin were indeed slowing down the DCPD to OCP transformation. Chu et al. [35] studied the influence of deliberately added (0.05 to 0.2 mM) amino acids (Gly, Ala, Ser, Asp, Glu, and His) on the solution-based phase transformation of DCPD at room temperature. Since Chu et al. [35] fixed the solution pH at 8.45, DCPD crystals placed into amino-acid containing solutions transformed only to apatite, and they reported that amino acids Gly, Ala, Ser and His were strongly inhibiting the

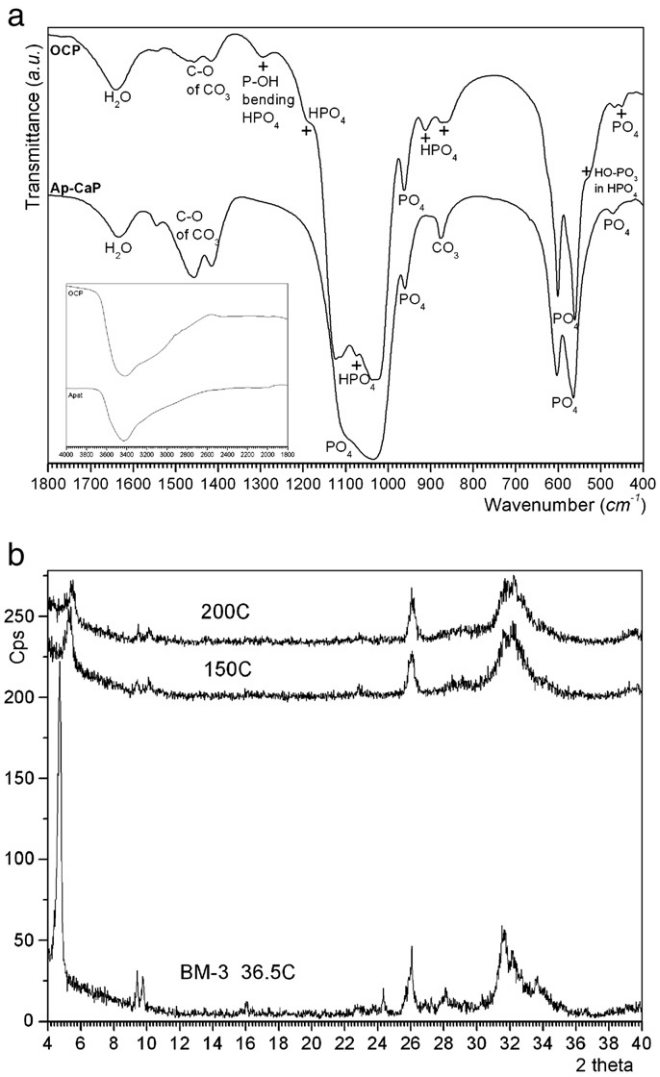

Fig. 6. a. Comparative FTIR chart of Ap-CaP (NH_4-DCPD, 85 °C, 1 h, stirred, HT-4) and OCP (NH_4-DCPD, 75 °C, 2.33 h, aged, HT-10) samples. b. Degradation of OCP by heating in air at 150 °C and 200 °C for 90 min (*in comparison to the OCP sample of BM-3-36.5 °C-aged-Na–K-DCPD-72 h sample*).

DCPD to apatite transformation at RT, mainly leaving DCPD unreacted in such solutions. OCP is not stable at pH 8.45, not at pH 7.4 as well. In our previous study [1], we have studied the transformation of DCPD in commercial DMEM solutions at 36.5 °C, and it must be noted that in DMEM solutions the concentrations of amino acids are significantly higher than those employed in the Chu et al. [35] study.

When Hepes was also eliminated in BM-3, it became possible for the first time to completely convert the DCPD crystals to OCP in less than 72 h at 36.5 °C (Fig. 2a). The effect of the presence of Hepes could be due to the complexation of some of the Ca^{2+} ions of the solution by the Hepes buffer at the Ca/P molar ratio of 1.99. This fact was previously reported by Serro and Saramago [36]. The buffering agent would form complexes with several cations, including Ca^{2+}, which would then reduce the concentration of free Ca^{2+} ions available for the real time DCPD to OCP transformation according to the reaction (2) above. This would also explain why BM-8 media (Ca/P molar ratio of 2.5) were able to successfully complete the DCPD to OCP transformation in less than 72 h although they contained Hepes (in comparison to BM-7, for instance). BM-8 had a Ca/P molar ratio of 2.5 and it thus had more non-complexed, free Ca^{2+} available. At higher Ca/P molar ratios "the inorganic backbone of a DMEM solution," i.e., BM-3 (1.99), and its Ca-enriched version, i.e., BM-7 (2.5), would perform well in facilitating the DCPD to OCP transformation.

Mg^{2+} ions are known [37] to partially retard the nucleation and growth of the crystals of apatitic calcium phosphate and for this reason their presence in these biomineralization media was deemed to be necessary, however, the elimination of magnesium in passing from BM-3 to BM-4 media did not cause any noticeable change in the DCPD to OCP transformation. This was expected since all the other remaining conditions (Ca/P ratio, HCO_3^- concentration, pH stability or instability, and the use of $NaH_2PO_4 \cdot 2H_2O$ as the phosphate ion source) of these media were in favor of the OCP formation at 36.5 °C, and only the elimination of Mg^{2+} ions would certainly not cause the conversion of DCPD, for instance, into apatitic CaP at that low temperature (Fig. 2a and b).

The complete removal of $NaHCO_3$ from the biomineralization media preparation, as in the cases of BM-5 and BM-6 was naturally the worst-case and the conversion to OCP was found to be minimal even after 72 h at 36.5 °C. The biomineralization media developed in this study, especially when they did not contain any Hepes (BM-3, BM-4 and BM-7), were largely based on the action of the CO_2/HCO_3^- buffering [38], and this was why these solutions contained exactly identical HCO_3^- concentration (i.e., 44.05 mM) with that of the DMEM solutions. As it is well known, one cannot find any Hepes or Tris in blood, but it instead contains a mixture of buffer systems CO_2/HCO_3^-, $H_2PO_4^-/HPO_4^{2-}$ and protein/Hprotein [38,39]. Therefore, the biomineralization media developed here did not need any Hepes or Tris, but they relied on the buffering action of the first two of the buffer systems mentioned above. In the case of complete removal of $NaHCO_3$ from the solutions, i.e., BM-5 and BM-6, therefore, it was not possible to transform DCPD into OCP. The small amounts of transformation achieved in the samples of BM-5 and BM-6 (Fig. 2c) in 72 h at 36.5 °C were only due to the buffering action of $H_2PO_4^-/HPO_4^{2-}$.

4.3. Accelerated transformation at temperatures >36.5 °C

The selection of BM-3 media, over those of BM-4 or BM-7, for the high-temperature (55 to 90 °C) experiments was therefore based on its similarity (the presence of Mg^{2+} ions and Ca/P molar ratio = 1.99) to the DMEM solutions. Stirring the media with the DCPD powders, while increasing the temperature, increased the transformation rate. The experimental results given in Table 2 showed that even increasing the temperature from 55 °C to 60 °C in a 1 h stirred experiment increased the chances of obtaining Ap-CaP. This was mainly due to the high solubility of DCPD, and once it started to dissolve (with the help of increased temperature), it re-precipitated as Ap-CaP. OCP is a metastable phase and catching and freezing in of this metastable phase are quite difficult, if not impossible, in the high-temperature runs. Then, the choice was only increasing the temperature and not stirring the media containing the DCPD powders. We have found that heating the BM-3 media having DCPD at 75°–80 °C (typically for 2 h) was the optimum to achieve the complete transformation into OCP.

We must underline that OCP is itself not suitable, just like DCPD, for heating in an air atmosphere; it deteriorated into cryptocrystalline apatitic CaP as shown in Fig. 6b. The samples of OCP (produced in BM-3 at 36.5 °C, 72 h) were heated in clean watch glasses at 150 and 200 °C for 90 min, followed by collecting the XRD data shown in Fig. 6b. Both samples exhibited around 9 to 9.2% weight loss and the (010) reflection of the OCP phase, which in pure OCP it is observed at 4.72° 2θ, decreased in intensity and shifted towards 5.33° 2θ after 150 °C heating and to 5.55° 2θ after being heated at 200 °C. Such a gradual deterioration of OCP with an increase in calcination temperature was interesting and warrants further study.

5. Conclusions

New biomineralization media were developed to facilitate the simple and rapid transformation of brushite (DCPD) into octacalcium

phosphate (OCP) over the temperature range of 36.5 to 80 °C within hours.

The use of either sodium- and potassium phosphate solutions or ammonium phosphate solutions in the synthesis of DCPD powders did not influence their transformation into OCP in the media developed and experimental conditions stated in this study. Spectator ions present in the DCPD synthesis solutions, such as Na^+, K^+ and/or NH_4^+, did not result in a noticeable change in the XRD, FTIR and SEM data.

To answer the questions posed at the start of this project, which was a direct follow-up of a previously published study of ours performed with a DMEM solution [1]; the amino acids, vitamins, glucose and Hepes present in a typical DMEM solution were found to be not essential to transform DCPD into OCP according to the findings of this study.

The deletion of magnesium ions from the biomineralization media did not influence the DCPD to OCP transition. However, the bicarbonate ions were essential for that transformation since they were mainly responsible from the pH buffering action.

Ca/P molar ratio of the biomineralization media did not adversely affect the transformation process, when it was increased from 1.99 to 2.50.

Finally, it was found that increasing the process temperature from the human body temperature of 36.5 °C to around 75 °C to 80 °C reduced the time of transformation of DCPD into OCP drastically from 72 to 2 h.

Acknowledgments

Authors are grateful for the help offered by the technician Kamelya Tatlidil of the Department of Genetics and Bioengineering of Yeditepe University in capturing the SEM images and Res. Assist. Demet Us of the College of Pharmacy of Yeditepe University in facilitating access to the FTIR.

We also would like to cordially thank the unknown reviewers of this manuscript who meticulously detected a number of crucial points which we happened to overlook in the writing stage.

Notes: Certain commercial equipment, instruments, or chemicals are only identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the authors, nor does it imply that the equipment or materials identified are necessarily the best available for the purpose.

References

[1] S. Mandel, A.C. Tas, Mat. Sci. Eng. C 30 (2010) 245.
[2] H.S. Azevedo, I.B. Leonor, C.M. Alves, R.L. Reis, Mat. Sci. Eng. C 25 (2005) 169.
[3] F. Yang, J.G.C. Wolke, J.A. Jansen, Chem. Eng. J. 137 (2008) 154.
[4] J.A. Juhasz, S.M. Best, A.D. Auffret, W. Bonfield, J. Mater. Sci. Mater. Med. 19 (2008) 1823.
[5] D. Bayraktar, A.C. Tas, J. Eur. Ceram. Soc. 19 (1999) 2573.
[6] R. Tang, M. Hass, W. Wu, S. Gulde, G.H. Nancollas, J. Colloid. Interf. Sci. 260 (2003) 379.
[7] L.J. Shyu, L. Perez, S.L. Zawacki, J.C. Heughebaert, G.H. Nancollas, J. Dent. Res. 62 (1981) 398.
[8] F.C.M. Driessens, R.M.H. Verbeeck, Biominerals, CRC Press, Boca Raton, FL, 1990, p. 38.
[9] B. Flautre, C. Maynou, J. Lemaitre, P. Van Landuyt, P. Hardouin, J. Biomed. Mater. Res. 63B (2002) 413.
[10] M. Bohner, F. Theiss, D. Apelt, W. Hirsiger, R. Houriet, G. Rizzoli, E. Gnos, C. Frei, J.A. Auer, B. von Rechenberg, Biomaterials 24 (2003) 3463.
[11] M. Bohner, U. Gbureck, J.E. Barralet, Biomaterials 26 (2005) 6423.
[12] R.M. Shelton, Y. Liu, P.R. Cooper, U. Gbureck, M.J. German, J.E. Barralet, Biomaterials 27 (2006) 2874.
[13] J.V. Rau, V.S. Komlev, A. Generosi, M. Fosca, V.R. Albertini, S.M. Barinov, J. Cryst. Growth 312 (2010) 2113.
[14] O. Suzuki, S. Kamakura, T. Katagiri, M. Nakamura, B. Zhao, Y. Honda, R. Kamijo, Biomaterials 27 (2006) 2671.
[15] T. Kikawa, O. Kashimoto, H. Imaizumi, S. Kokubun, O. Suzuki, Acta Biomater. 5 (2009) 1756.
[16] Y. Murakami, Y. Honda, T. Anada, H. Shimauchi, O. Suzuki, Acta Biomater. 6 (2010) 1542.
[17] O. Suzuki, Acta Biomater. 6 (2010) 3379.
[18] T. Keshima, M. Sakurai, S. Lee, K. Yubuta, S. Ito, T. Suzuki, T. Shishido, M. Endo, S. Oishi, Cryst. Growth Des. 9 (2009) 650.
[19] M. Kumar, J. Xie, K. Chittur, C. Riley, Biomaterials 20 (1999) 1389.
[20] J. Redepenning, G. Venkataraman, J. Chen, N. Stafford, J. Biomed. Mater. Res. 66A (2003) 411.
[21] X. Cheng, M. Filiaggi, S.G. Roscoe, Biomaterials 25 (2004) 5395.
[22] A.C. Tas, Int. J. Mater. Res. 97 (2006) 639.
[23] A.C. Tas, S.B. Bhaduri, J. Am. Ceram. Soc. 87 (2004) 2195.
[24] G. Girisken, Development of a New Biomineralization/Calcification Solution, Senior Capstone Project, Dept. of Biomedical Eng, Yeditepe University, Istanbul, Turkey, January 2010.
[25] N. Temizel, Rapid Transformation of Brushite to OCP at Temperatures between 36.5° and 80 °C, Senior Capstone Project, Dept. of Biomedical Eng., Yeditepe University, Istanbul, Turkey, June 2010.
[26] R.Z. LeGeros, Calcified Tissue Int. 37 (1985) 194.
[27] R.Z. LeGeros, G. Daculsi, I. Orly, T. Abergas, W. Torres, Scanning Microsc. 3 (1989) 129.
[28] M. Markovic, B.O. Fowler, W.E. Brown, Chem. Mater. 5 (1993) 1406.
[29] J. Xu, I.S. Butler, D.F.R. Gilson, Spectrochim. Acta A 55 (1999) 2801.
[30] M.S. Tung, L.C. Chow, W.E. Brown, J. Dent. Res. 64 (1985) 2.
[31] L. Perez, L.J. Shyu, G.H. Nancollas, Colloid. Surface 38 (1989) 295.
[32] J.E. Eastoe, Biochem. J. 61 (1955) 589.
[33] C. Combes, C. Rey, M. Freche, J. Mater. Sci. Mater. Med. 10 (1999) 153.
[34] G.H. Nancollas, M. Lore, L. Perez, C. Richardson, S.J. Zawacki, Anat. Rec. 224 (1989) 234.
[35] X. Chu, W. Jiang, Z. Zhang, Y. Yan, H. Pan, X. Xu, R. Tang, J. Phys. Chem. B 115 (2011) 1151.
[36] A.P. Serro, B. Saramago, Biomaterials 24 (2003) 4749.
[37] F. Barrere, C.A. van Blitterswijk, K. de Groot, P. Layrolle, Biomaterials 23 (2002) 2211.
[38] P.A.A.P. Marques, M.C.F. Magalhaes, R.N. Correira, Biomaterials 24 (2003) 1541.
[39] J. Baggot, Textbook of Biochemistry with Clinical Correlations, Wiley, New York, 1997, p. 1031.