Materials Science and Engineering C 30 (2010) 245–254

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Brushite ($CaHPO_4 \cdot 2H_2O$) to octacalcium phosphate ($Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$) transformation in DMEM solutions at 36.5 °C

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ARTICLE INFO

Article history:
Received 23 September 2009
Accepted 26 October 2009
Available online 30 October 2009

Keywords:
Calcium phosphate
Brushite
Transformation
DMEM

ABSTRACT

The purpose of this study was to investigate the transformation of brushite (dicalcium phosphate dihydrate, DCPD, $CaHPO_4 \cdot 2H_2O$) powders at 36.5 °C in DMEM (Dulbecco's Modified Eagle Medium) solutions. Two sets of brushite powders with different particle shapes were synthesized to use in the above DMEM study. The first of these brushite powders was prepared by using a method which consisted of stirring calcite ($CaCO_3$) powders in a solution of ammonium dihydrogen phosphate ($NH_4H_2PO_4$) from 6 to 60 min at room temperature. These powders were found to consist of dumbbells of water lily-shaped crystals. The second one of the brushite powders had the common flat-plate morphology. Both powders were separately tested in DMEM-immersion experiments. Monetite (DCPA, $CaHPO_4$) powders were synthesized with a unique water lily morphology by heating the water lily-shaped brushite crystals at 200 °C for 2 h. Brushite powders were found to transform into octacalcium phosphate (OCP, $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$) upon soaking in DMEM (Dulbecco's Modified Eagle Medium) solutions at 36.5 °C over a period of 24 h to 1 week. Brushite powders were known to transform into apatite when immersed in synthetic (simulated) body fluid (SBF) solutions. This study found that DMEM solutions are able to convert brushite into OCP, instead of apatite.

1. Introduction

DMEM (Dulbecco's Modified Eagle Medium) solutions are used in cell culture as the growth medium. Hepes-buffered DMEM solutions contain inorganic salts (to supply Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}, Fe^{3+}, $H_2PO_4^{-}$, HCO_3^{-} and Cl^{-} ions), amino acids, vitamins and glucose. DMEM solutions have a Ca/P molar ratio of 1.99.

SBF (simulated [1] or synthetic body fluid [2]) solutions, on the other hand, are usually Tris- or Hepes-buffered [3] and only contain inorganic salts with an overall Ca/P molar ratio of 2.50.

SBF solutions cannot be used in cell culture studies since they lack the necessary nutrients, such as amino acids, vitamins and glucose, to allow and sustain the proliferation of living organisms.

However, SBF solutions were heavily used in recent decades to test the so-called bioactivity of a given material, regardless of being metallic, ceramic, glassy or polymeric. Can bioactivity be tested *in vitro* with SBF solution [4]? How useful is SBF in predicting *in vivo* bone bioactivity [5]? These two questions were previously asked and had actually become the exact titles of two articles cited here [4,5].

Nevertheless, the following question has not been asked frequently: could it be possible to test, *in vitro*, such a so-called bioactivity by using readily available DMEM solutions, containing amino acids, vitamins and glucose, instead of SBFs? The current study asks this question in search of an answer to it, by ageing brushite (dicalcium phosphate dihydrate, DCPD, $CaHPO_4 \cdot 2H_2O$) powders in DMEM solutions at 36.5 °C.

Two different, chemically-synthesized $CaHPO_4 \cdot 2H_2O$ powders with quite diverse particle morphologies were tested by soaking them in DMEM solutions at the human body temperature of 36.5 °C from 1 day to 1 week.

Brushite is a relatively high solubility (with log K_{sp} of −6.6 [6]) calcium phosphate compound and is known to convert into apatite-like calcium phosphate when soaked in SBF solutions at the human body temperature for about one week [7–21].

However, both of the brushite powders used in this study were found to transform into octacalcium phosphate (OCP, $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$) when soaked in DMEM solutions.

To the best of our knowledge, this study could be the first to report on the bulk transformation of brushite powders into OCP upon soaking in a common cell culture solution, such as DMEM.

2. Materials and methods

2.1. Synthesis of brushite or monetite with water lily-shaped (WL) crystals

Brushite (DCPD, $CaHPO_4 \cdot 2H_2O$) powders with a unique water lily morphology were synthesized as follows. 40.0 g (=0.3477 mol P) of ammonium dihydrogen phosphate, $NH_4H_2PO_4$ (≥99.9%, Cat. No.: 1.01126, Merck KGaA, Darmstadt, Germany) was first dissolved in 340 mL of distilled water. This solution had a pH value of 3.9 ± 0.1 at

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doi:10.1016/j.msec.2009.10.009

room temperature (RT). The solution was placed into a 500 mL-capacity Pyrex glass media bottle. 10.0 g (=0.0999 mol Ca) of calcite, $CaCO_3$ (≥99.9%, Cat. No.: 12010, Riedel-de-Haen, Germany) powder was added into the bottle. The formed suspension was stirred (500 rpm) at RT for 30 min, by using a Teflon-coated magnetic stirrer. After 30 min of stirring, the pH value of the white suspension was measured to be 5.9 ± 0.1. The particles of the suspension were recovered from their mother liquor by using a porcelain Buechner funnel containing a No. 3 Whatman filter paper. The funnel was attached to a mechanical vacuum pump during filtration. The wet cake on the filter paper was finally washed with 750 mL of distilled water. Obtained powders were dried in a clean watch-glass overnight at 75 °C, in a static air microprocessor-controlled oven, to obtain 14.64 ± 0.2 g of brushite with the water lily (WL, *Nymphaeaceae*) morphology.

The above was the optimized and up-scaled synthesis recipe of the WL-shaped brushite powders. Prior to the development of this recipe, the influence of stirring time on the advance of reaction was studied over the range of 90 s to 60 min. In these experiments, 10.0 g of $NH_4H_2PO_4$ was first dissolved in 85 mL of distilled water and then stirred at RT with 2.5 g of calcite ($CaCO_3$) powder in 100 mL-capacity glass bottles containing 85 mL distilled water.

To convert the WL-shaped brushite crystals into monetite (DCPA, $CaHPO_4$), 1.0 g of the above brushite powders was kept (in clean watch glasses) for 2 h in a microprocessor-controlled static air oven pre-heated to 200 °C. The following reaction was expected to take place during this heating:

$$CaHPO_4 \cdot 2H_2O(s) \rightarrow CaHPO_4(s) + 2H_2O(g). \quad (1)$$

2.2. Synthesis of brushite with flat-plate-shaped (FP) crystals

The synthesis procedure used to form 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.: 1.04873, Merck KGaA) was dissolved in 700 mL of distilled water, followed by the addition of 3.013 g of Na_2HPO_4 (≥99.9%, Cat. No.: 1.06586, Merck KGaA), which resulted in a clear solution of pH 7.5 at RT. Solution B (of pH 6.4) was prepared by dissolving 4.014 g of $CaCl_2 \cdot 2H_2O$ in (≥99.9%, Cat. No.: 1.02382, Merck KGaA) 200 mL of 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 from their mother liquors were dried overnight at 65 °C to obtain 3.28 g of FP-shaped brushite powders.

2.3. Transformation of WL-shaped brushite into OCP in DMEM solution

Glass media bottles (100 mL-capacity) containing 50 mL of DMEM solutions (DMEM, High glucose 1×, Sterile, Product No.: 21063-029, Gibco, Invitrogen, USA) were used. Table 1 showed the composition of the DMEM solutions of this study. 1.0 g of WL-shaped 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 36.5 ± 0.1 °C. The times for ageing the brushite powders in DMEM solutions were selected as 24, 48 h and one week. The DMEM solution of the one week sample was replenished with a fresh solution after 120 h. At the end of the specified ageing periods, the solids were recovered from the solutions by using a porcelain Buechner funnel and No. 2 Whatman filter paper, with vacuum filtration. The solids were washed with 500 mL of distilled water. Washed samples were left to dry overnight at 65 °C to finally obtain DMEM-transformed powders with the following weights: 0.80 ± 0.03 g, 0.81 ± 0.03 g, and 0.70 ± 0.03 g for the 24 h-, 48 h- and one week-aged samples, respectively.

Table 1
Composition of the DMEM solution used in this study.

Component	Concentration (mM)
Amino acids	
Glycine	0.4
L-Arginine hydrochloride	0.398
L-Cystine 2HCl	0.201
L-Glutamine	4
L-Histidine hydrochloride-H_2O	0.2
L-Isoleucine	0.802
L-Leucine	0.802
L-Lysine hydrochloride	0.798
L-Methionine	0.201
L-Phenylalanine	0.4
L-Serine	0.4
L-Threonine	0.798
L-Tryptophan	0.0784
L-Tyrosine disodium salt	0.398
L-Valine	0.803
Vitamins	
Choline chloride	0.0286
D-Calcium pantothenate	0.00839
Folic acid	0.00907
Niacinamide	0.0328
Pyridoxine hydrochloride	0.0194
Riboflavin	0.00106
Thiamine hydrochloride	0.0119
I-Inositol	0.04
Inorganic salts	
Calcium chloride anhyd.	1.8
Ferric nitrate ($Fe(NO_3)_3 \cdot 9H_2O$)	0.000248
Magnesium sulfate ($MgSO_4$) anhyd.	0.814
Potassium chloride (KCl)	5.33
Sodium bicarbonate ($NaHCO_3$)	44.05
Sodium chloride (NaCl)	81.9
Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$)	0.906
Other components	
D-Glucose (dextrose)	25
Hepes	25.03

From: http://www.invitrogen.com.

2.4. Transformation of FL-shaped brushite into OCP in DMEM solution

Glass media bottles (100 mL-capacity) containing 50 mL of DMEM solutions (DMEM, High glucose 1×, Sterile, Product No.: 21063-029, Gibco, Invitrogen, USA) were used. 0.65 g of FP-shaped 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 36.5 ± 0.1 °C. The times for ageing the FL-type brushite powders in DMEM solutions were selected as 48 h and one week. The DMEM solution of the one week sample was replenished with a fresh solution at every 48 h. At the end of the specified ageing periods, the solids were recovered from the solutions by using a porcelain Buechner funnel and No. 2 Whatman filter paper, with vacuum filtration. The solids were washed with 500 mL of distilled water. Washed samples were left to dry overnight at 65 °C to finally obtain DMEM-transformed powders with the following weights: 0.46 ± 0.01 g and 0.45 ± 0.01 g for the 48 h- and one week-aged samples, respectively.

2.5. Sample characterization

All powder samples were characterized by using a powder X-ray diffractometer (Advance D8, Bruker AG, Karlsruhe, Germany) after being lightly grinded by 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.

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.

Fourier-transform infrared spectroscopy (Spectrum One, Perkin Elmer, USA) analyses were performed after mixing 1 mg of sample powders with 300 mg of KBr powder, 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.

3. Results

The X-ray diffraction (XRD) and Fourier-transform infrared (FTIR) spectra of the two different brushite powders (denoted as FP and WL powders) synthesized in this study are given in Fig. 1a. The XRD spectra given in Fig. 1b depicted that for the synthesis of WL-shaped brushite crystals, 90 s of mixing was not enough and the calcite powders remained still unreacted after 90 s, but in a time of stirring as short as 6 min brushite was forming in the aqueous $CaCO_3$–$NH_4H_2PO_4$ suspensions. Therefore, the selection of a mixing time between 6 and 60 min would be appropriate. There were no changes in the crystal size and shape of WL-type brushite powders if one increased the mixing time from 6 to 30 min or from 30 to 60 min. The scanning electron photomicrographs (SEM) of both WL and FP powders were shown in Fig. 1c.

FP (flat-plate) powders consisted of large and flat plates of brushite, with a preferred growth along the (*020*) crystallographic planes and the relatively high X-ray intensities obtained from these planes were apparent in the top XRD trace of Fig. 1a. Those flat plates were found to be thin (about 150 nm), had a width between 5 and 10 μm, and could elongate to about 70 to 80 μm. Flat-plate morphology is quite common to the precipitated brushite powders and could be frequently encountered in the literature on brushite. On the other hand, the WL (water lily, *Nymphaeaceae*) brushite powders of this study comprised dumbbells of water lily-shaped crystals. These crystals were again large, extending to about 80–100 μm. For the WL-type brushite crystals (in comparison to the FP powders) almost equal X-ray intensities for the (*020*) and (*1 2 −1*) planes were registered as seen in Fig. 1a; i.e., the bottom XRD trace.

The FTIR inset shown in Fig. 1a disclosed that the WL powders contained a certain amount of unreacted $CaCO_3$, which was not detected by the XRD spectra of the same powders. The starting powder for the WL-type brushite was precipitated $CaCO_3$ spindle-shaped particles. The SEM morphology of the $CaCO_3$ powders used in the current study was given elsewhere [22]. During the synthesis of WL-type brushite, these $CaCO_3$ (calcite) particles were chemically attacked by the acidic $H_2PO_4^-$ ions in solution, and a templated synthesis-type reaction followed that. The calcite particles were behaving as the template and the dumbbells of stacked brushite water lilies gradually formed in place of the original template. Since this was a room temperature aqueous reaction starting from the surface of the calcite template particles and advancing with time toward the cores of particles, it could be regarded as reasonable to have very small amounts (i.e., not detectable by the X-rays) of unreacted $CaCO_3$ at their cores. Such WL-shaped brushite crystals, to the best of our knowledge, were not reported prior to this study.

The XRD, FTIR and SEM data given in Fig. 2 denoted that the monetite ($CaHPO_4$) powders obtained from the 200 °C-heating (for 2 h) of WL-brushite powders were single-phase and still preserved the WL morphology. Brushite-to-monetite conversion follows a simple dehydration reaction. The theoretical weight loss in the brushite-to-monetite conversion is 20.94%, which represents the loss of two water molecules. Our 200 °C-heating runs always exhibited around 21% weight loss. Monetite powders with this WL-type particle morphology, to the best of our knowledge, were again not reported before. Commercially available monetite powders typically comprise rectangular prismatic or cubic particles (whose SEM morphology was reported elsewhere [23]).

Brushite crystallizes in the monoclinic space group *Cc* with the lattice parameters $a=6.359$, $b=15.177$, $c=5.81$ Å, and $\beta=118.54°$ [24]. Triclinic monetite has the following lattice parameters: $a=6.910$, $b=6.627$, $c=6.998$ Å, $\alpha=96.34°$, $\beta=103.82°$, and $\gamma=88.33°$ [24]. The experimentally-determined lattice parameters of brushite and monetite powders synthesized in this study differed very slightly (only in the third decimal place) from the above-mentioned literature values.

The analysis of FTIR data reproduced in the inset of Fig. 1a revealed the following IR frequencies (in cm^{-1}). The bands at 3544, 3491, 3290, and 3163 were due to the O–H stretching of water. The shoulder at 2955 was again that of O–H stretching. H_2O bending was recorded at 1653 cm^{-1}. The O–H in-plane bending was measured at 1219 cm^{-1}. PO stretching was observed at 1134, 1057, and 987 cm^{-1}. P–O(H) stretching was found at 876 cm^{-1} for the FP (flat plate) sample, however, its shift to 871 cm^{-1} in the WL (water lily) sample together with the appearance of a carbonate band at 1472 cm^{-1} was indicative of unreacted calcite presence in the WL samples. H_2O libration was observed in both samples at 791 cm^{-1}. Finally, PO bending was recorded at 662, 576, and 525 cm^{-1}. The observed IR band positions (of Figs. 1a and 2) were in close agreement with those reported by Xu et al. [25].

Both brushite powders were largely transformed into octacalcium phosphate (OCP, $Ca_8(HPO_4)_2(PO_4)_4\cdot 5H_2O$) upon one week of soaking in DMEM solution at 36.5 °C, as shown by the X-ray diffraction data of Fig. 3a. WL samples still contained some unreacted brushite phase after 1 week in DMEM, but the FP samples were able to completely transform into OCP. Even after 48 h of ageing (pH dropping from the initial 7.4 to around 6.8) in the non-replenished DMEM solutions, OCP was the major phase in the FP samples. The lattice parameters of the triclinic OCP phase determined from the FP-1 week sample (Fig. 3a, top trace) were $a=9.530$, $b=18.991$, $c=6.854$ Å, $\alpha=92.30°$, $\beta=90.11°$, and $\gamma=79.94°$, and they were in close agreement with those reported in ICDD PDF 026-1056 [26]. The FTIR data of the above samples were depicted in Fig. 3b, which were in good agreement with those previously reported by Wu and Nancollas [27], Suzuki et al. [28], LeGeros et al. [29], and LeGeros [30].

The following IR bands were observed in the FTIR spectrum of FP-1 week sample shown in Fig. 3b. H–O–H or crystalline water of OCP was assigned to the wide band recorded over the range of 3700–3000 cm^{-1}. H_2O bending was at 1647 cm^{-1}. The P–OH bending modes originating from the HPO_4 groups of OCP were observed at 1296 and 1193 cm^{-1}. P–O in HPO_4 and PO_4 groups were recorded at 1126, 1110, 1075, 1058, 1040, 1023, 962, 628, 603, 560, 471, and 452 cm^{-1}. The P–OH stretching mode of HPO_4 groups was at 914 and 874 cm^{-1}. Finally, the HO–PO_3 bending mode in HPO_4 was found at 525 cm^{-1}. The IR band assignments of the FP-1 week samples of this study were in good agreement with those reported by LeGeros et al. [29].

The changes that occurred in the particle morphology of brushite powders aged in DMEM solutions at 36.5 °C were followed by the SEM photomicrographs given in Fig. 4a through d. It was found that one week was enough to fully convert the brushite crystals into octacalcium phosphate, as also supported by the FTIR and XRD data.

The DMEM solution used in this study was shown to be a convenient and robust medium to synthesize OCP powders in a static glass medium bottle, heated at 36.5 °C, by starting with brushite powders synthesized in this study. It must be remembered that brushite powders soaked in SBF solutions at 37 °C, under similar conditions, were only transforming into apatite [7–21].

In the current study, the DMEM solutions aged alone (i.e., without any brushite powders) in clean and sealed glass media bottles at 37 °C, for one week, did not produce any visible precipitates within the bottles. This finding indicated that the DMEM solutions of this study were not autogenously crystallizing the OCP phase.

4. Discussion

The major question resulting from this study is the following. If the brushite crystals soaked in Hepes-buffered DMEM solutions transform into OCP, whereas brushite crystals soaked in Tris-buffered SBF solutions transform into HA as reported in the previous studies [7–21], then which medium (DMEM or SBF) one needs to rely on as the correct bioactivity test for at least the brushite powders?

Octacalcium bis(hydrogenphosphate) tetraphosphate pentahydrate, typically referred to as octacalcium phosphate (OCP, $Ca_8(HPO_4)_2(PO_4)_4{\cdot}5H_2O$), was proposed by Brown [31,32] to be the precursor during the formation of apatitic calcium phosphate phase in teeth enamel mineralization and also in bone formation. OCP is a thermodynamically metastable phase with respect to hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) [33], and the transformation from OCP to HA usually takes place rapidly, which is governed by the solution factors such as ion concentrations and pH. The log K_{sp} values of OCP and HA phases are −49.3 [34] and −117.1 [6], respectively. This significant difference between the solubilities of OCP and HA explains well why the mineralized portion of human hard tissues cannot be made of OCP alone. It seems like nature uses a transient

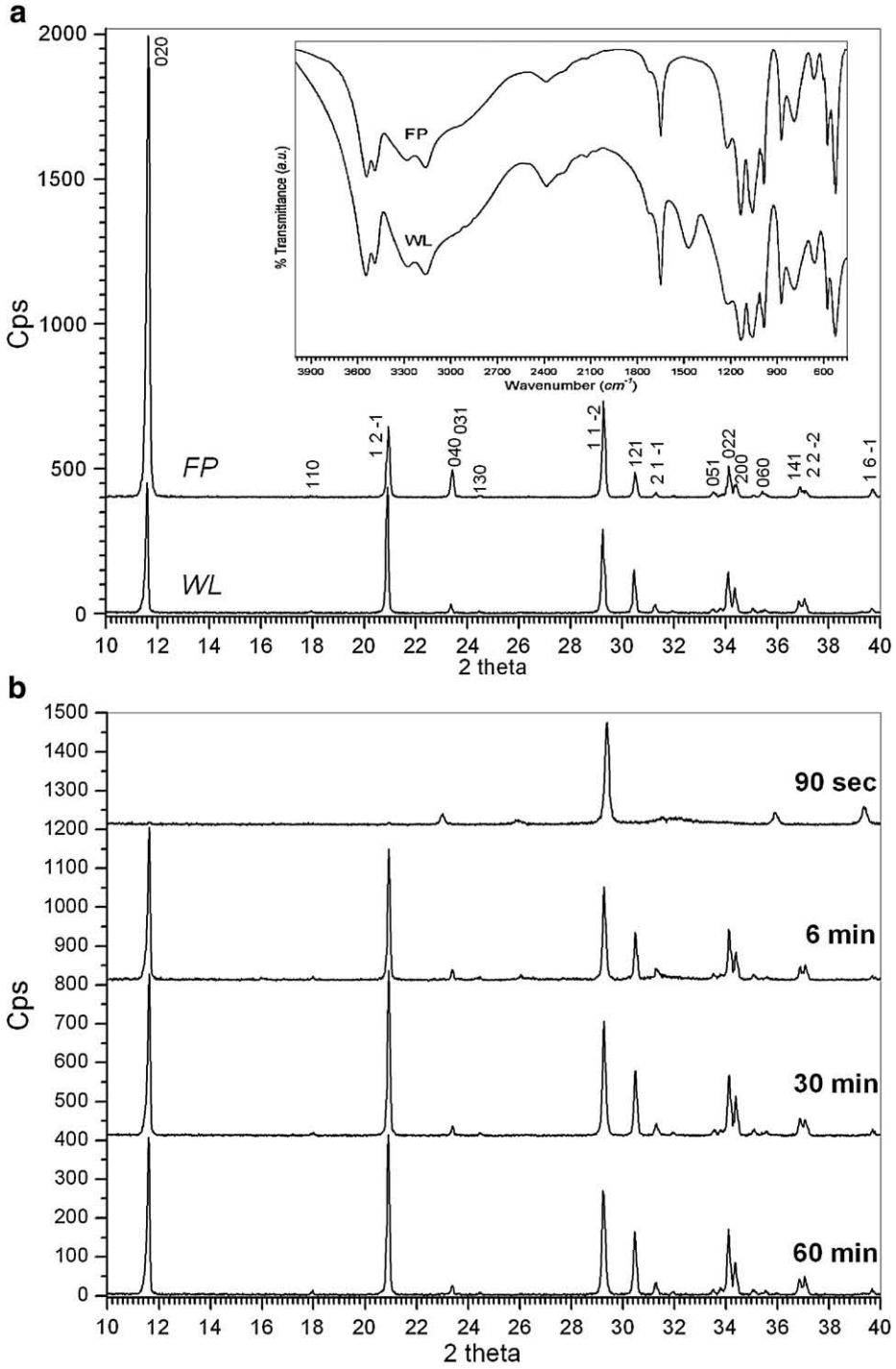

Fig. 1. a. XRD spectra of WL- (30 min stirring) and FP-type brushite powders; inset is depicting the FTIR spectrum of WL (30 min) and FP-type brushite. b. XRD spectra of WL samples as a function of stirring time; 90 s trace indicated single-phase calcite, while the others belonged to single-phase brushite. c. SEM photomicrographs of FP (background) and WL (inset) brushite crystals.

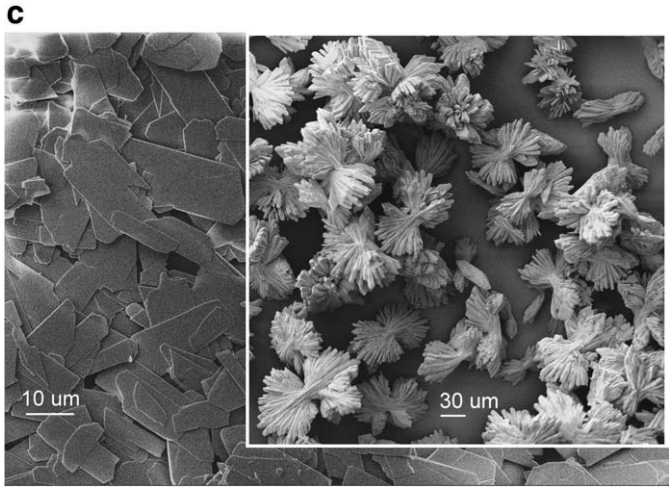

Fig. 1 (*continued*).

phase like OCP as the initial step in reaching to the more durable HA crystallites in her biological biomineralization and maturation processes.

One must take into account that implanted intraocular lenses exhibited (*ex vivo*) calcification phenomena in certain cases, and very interestingly the calcified phase was OCP, but not HA [35]. Guan et al. [35] reported that both silicone and fatty acids, such as myristic, palmitic, stearic, arachidic, and behenic, had important roles in inducing OCP nucleation and growth on silicone-treated intraocular lens surfaces. These authors unfortunately did not provide a satisfactory explanation why HA was not observed *in lieu* of OCP.

Brushite transforms into OCP by following the below reaction:

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

OCP is a more basic calcium phosphate phase in comparison to brushite, and OCP shall further hydrolyze to convert itself into HA. The transformation of brushite into OCP should have been facilitated by heterogeneous nucleation and the role of possible crystal defects on the brushite surfaces could well be considered [36]. The generation of $H_2PO_4^-$ ions in reaction (2) would cause a readily measurable pH decrease [37]. This was why we observed a consistent pH decrease in our DMEM ageing solutions, from 7.4 to around 6.8 within the first 72–120 h of ageing. It must be remembered that the buffering capacity of Hepes [38,39] is not as strong as that of Tris [40].

Another question comes up here. Do bone cells (i.e., osteoclasts, osteoblasts, etc.) always function and proliferate at a stable pH of 7.3 to 7.4 while they participate in the remodeling of bones? The answer to this question is negative and it is known that bone resorption (the first leg of healthy bone remodeling) is increased in metabolic acidosis [41], which can be mimicked experimentally by maintaining the cells at the extracellular pH of 6.5. Schilling et al. [42], for instance, found a more than four-fold increase in the number of osteoclasts compared to physiologic pH in a slightly acidic culture environment with an optimum between pH 6.9 and pH 7.1.

On the other hand, in uncorrected acidosis, the deposition of alkaline mineral (i.e., HA) in bone by osteoblasts (the second leg of healthy bone remodeling) is reduced, and osteoclast resorptive activity is increased in order to maximize the availability of OH^- ions in solution to counteract protons. Osteoblast alkaline phosphatase (ALP) activity peaks near the physiologic pH 7.4, but was found by Brandao-Burch et al. [43] to be reduced eight-fold at around pH 6.9. The same pH reduction is associated with two- and four-fold increases in Ca^{2+} and HPO_4^{2-} solubility for hydroxyapatite, respectively [43]. For more detailed analysis of the influence of medium pH on the activity of human osteoblasts in culture (such as alkaline phosphatase activity, lactate production, proline hydroxylation, DNA content and thymidine incorporation), interested readers may consult the article by Kaysinger and Ramp [44].

If, for example, a Tris-buffered SBF solution cannot allow its pH to drop from 7.4 to around 6.5–6.9, then it could not be possible for such a solution to mimic any osteoclast resorptive activity. Such a solution seems to be only programmed to heterogeneously nucleate nano-textured HA crystallites on the immersed substrates which do not cause a shift in the solution pH value towards 6.5 to 7. Hydroxylated surfaces ease this HA nucleation, and the pre-soaking of titanium coupons in heated solutions of NaOH or KOH prior to the SBF immersion constitutes a good example.

Calcification in cell culture media is a well-known phenomenon. For example, de Jonge et al. [45] very recently reported that soaking titanium and calcium phosphate-coated titanium substrates in α-MEM (supplemented with fetal calf or bovine serum (FBS), ascorbic acid, glycerophosphate and gentamycin) solutions at 37 °C led to the formation of apatitic calcium phosphate calcification on those. However, de Jonge et al. [45] did not report the formation of OCP. The presence or absence of FBS in cell culture media exerts a significant difference on the calcification products.

There surely is a difference in the calcification potentials of α-MEM (minimum essentials medium Eagle, α-modification) and DMEM solutions, and this was best explored in the study of Coelho et al. [46] performed on the human osteoblastic cell cultures. DMEM is a less nutrient-rich medium with respect to amino acids and vitamins, although, nutrient concentrations are, on the whole, higher than those found in α-MEM [46]. α-MEM contains ascorbic acid, and less $NaHCO_3$ (27 mM) than that found in DMEM (44 mM). Coelho et al. [46] reported the formation of slightly higher amounts of apatitic calcium phosphate spherules (but not OCP) in DMEM solutions after culturing the osteoblasts for more than four weeks, by providing SEM photomicrographs and EDXS analysis results.

Interestingly, Price et al. [47] found that their calcification studies on rat aortas soaked in DMEM solutions alone were not able to form any calcification products; however, DMEM solutions supplemented with 1.5% human, bovine or rat blood serum were able to form apatitic calcification products easily on the same rat aortas. Price et al. [47] attributed this behavior to the presence of a potent serum calcification factor (i.e., the noncollagenous serum protein fetuin) in serum.

Therefore, in comparing the Coelho et al. [46] and Price et al. [47] studies one should realize that in the first study the DMEM solutions contained osteoblasts and the alkaline phosphatase (ALP) released

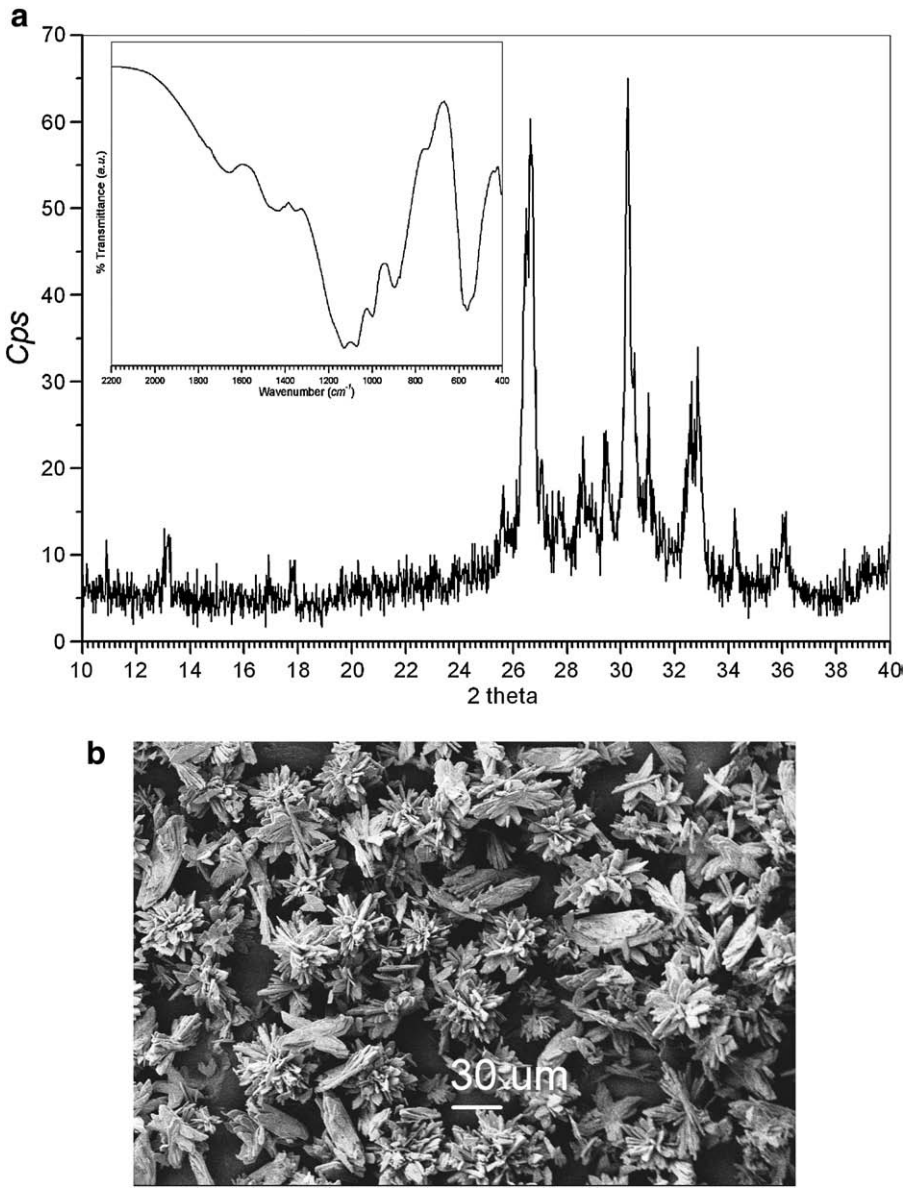

Fig. 2. a. XRD and FTIR (inset) spectra of monetite ($CaHPO_4$) powders with WL morphology. b. Characteristic SEM photomicrograph of monetite ($CaHPO_4$) powders with WL morphology.

from the osteoblasts would appear in the medium, whereas in the latter study there were no osteoblasts to secrete ALP and hence the serum calcification factors.

Until now, OCP crystallization was more or less considered to take place in aqueous solutions containing monocarboxylates, such as acetate (or formate) ions; this was probably due to the quite influential papers by Newesely [48] and LeGeros [30] on OCP synthesis. These synthesis procedures involved the mixing of calcium nitrate solutions with sodium acetate solutions or calcium acetate solutions with sodium phosphate solutions, respectively. LeGeros procedure envisaged the precipitation process to be performed between 60 and 80 °C [30]. Liu et al. [49] slightly modified the Newesely procedure of OCP synthesis, mixed calcium nitrate and disodium hydrogen phosphate solutions in a sodium acetate solution, continued the precipitation–maturation process at 45 °C for 48 h, but were not able to obtain single-phase OCP powders according to their FTIR spectra. Brown et al. [31] have historically been the first to hydrolyze brushite powders in a concentrated sodium acetate solution, and the same procedure was recently repeated by Monma et al. [50]. Our study, on the other hand, demonstrated a new route which totally eliminated the use of quite concentrated (0.2 M [49] or 0.5 M [50]) acetate solutions for synthesizing the OCP powders.

The absence of any previous studies related to the ageing/immersion of brushite crystals or powders, at the human body temperature of 36.5 °C, in a DMEM solution containing amino acids, the present authors are somewhat compelled to search for studies performed on titanium by using other calcification solutions.

Wen et al. [51] observed the formation of an OCP-like phase on the surface of HCl–H_2SO_4 and NaOH-treated commercially pure titanium immersed into a Tris/HCl-buffered and Mg- and HCO_3-free supersaturated calcification solution, SCS, having a Ca/P molar ratio of 1.67. This solution was, therefore, different from the popular SBF solutions also in terms of its Ca/P molar ratio and its degree of supersaturation with respect to apatite nucleation. The absence of Mg^{2+} and HCO_3^- ions in the solution described by Wen et al. [51] and the observation of OCP in place of single-phase apatite must be underlined. Wen and Moradian-Oldak [52] later reported the formation of OCP (instead of apatite) on titanium surfaces immersed in Mg- and HCO_3-free and Tris-buffered supersaturated calcification solutions (SCS) both without and with bovine serum

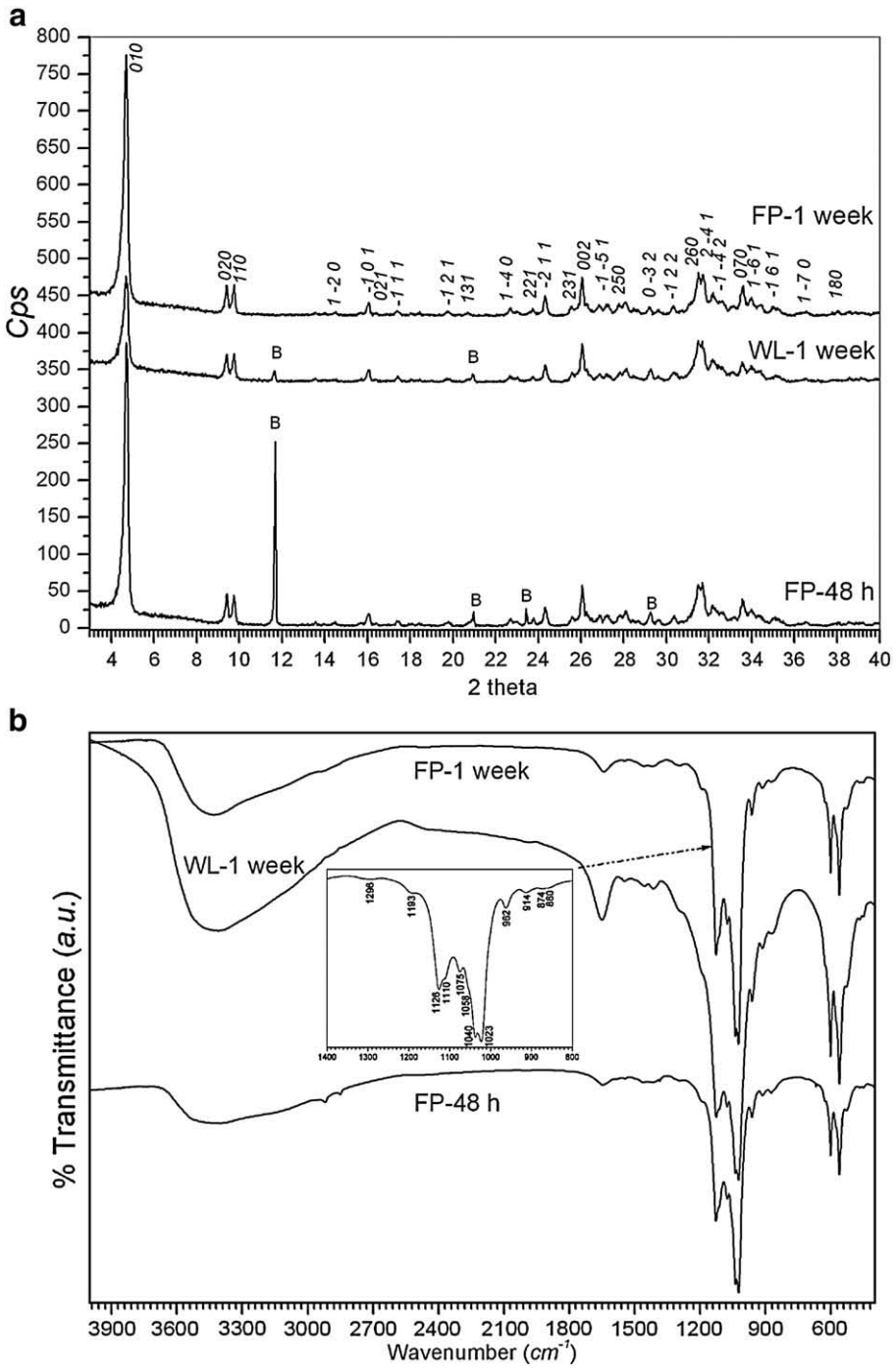

Fig. 3. a. XRD traces of WL- and FP-type brushite powders soaked in DMEM solution at 36.5 °C (times indicated soaking periods); FP-1 week trace showing single-phase OCP together with the crystallographic indices of the OCP phase; letter B indicated the brushite peaks. b. FTIR traces of WL- and FP-type brushite powders soaked in DMEM solution at 36.5 °C; the inset is showing the detail over the 1400 to 800 cm^{-1} range of the FP-1 week samples.

albumin or murine amelogenin. These two reports [51,52], contributed by the same first author, seemed to assert a significant difference in terms of the phase nature of the calcium phosphate deposited by the SCS (Mg- and HCO_3-free) and SBF (Mg- and HCO_3-containing) solutions. The same Mg- and HCO_3-free SCS solution, originally reported in the Wen et al. [51] paper, was then reproduced and reported in a number of seemingly follow-up studies to form OCP deposits on titanium [53,54] or HA or HA-TCP bioceramics [55].

However, the Hepes-buffered DMEM solution used in our study contained both Mg^{2+} and HCO_3^- ions, and was still able to completely transform the immersed brushite powders into OCP in 7 days. Therefore, the crystallization of OCP from a Tris-buffered SCS (without Mg and HCO_3) or Hepes-buffered DMEM solution cannot be simply attributed to or explained by the absence or presence of Mg^{2+} and HCO_3^- ions. The main question here should again be focused on the pH-stability of solutions buffered by using Tris or Hepes. Wen et al. [51] reported that the pH of their SCS solutions started from 7.4 and gradually dropped to around 7.2 within the first 16 h of immersion. This rapid drop in pH should be the reason for forming OCP instead of HA on their titanium coupons.

A possible answer to this issue was provided by an article of Serro and Saramago [56]. Serro and Saramago [56] prepared a new solution designated as SBF0, with the same composition of Kokubo SBF [1] but without the buffer Tris. The buffer Tris, present in SBF, is known to form

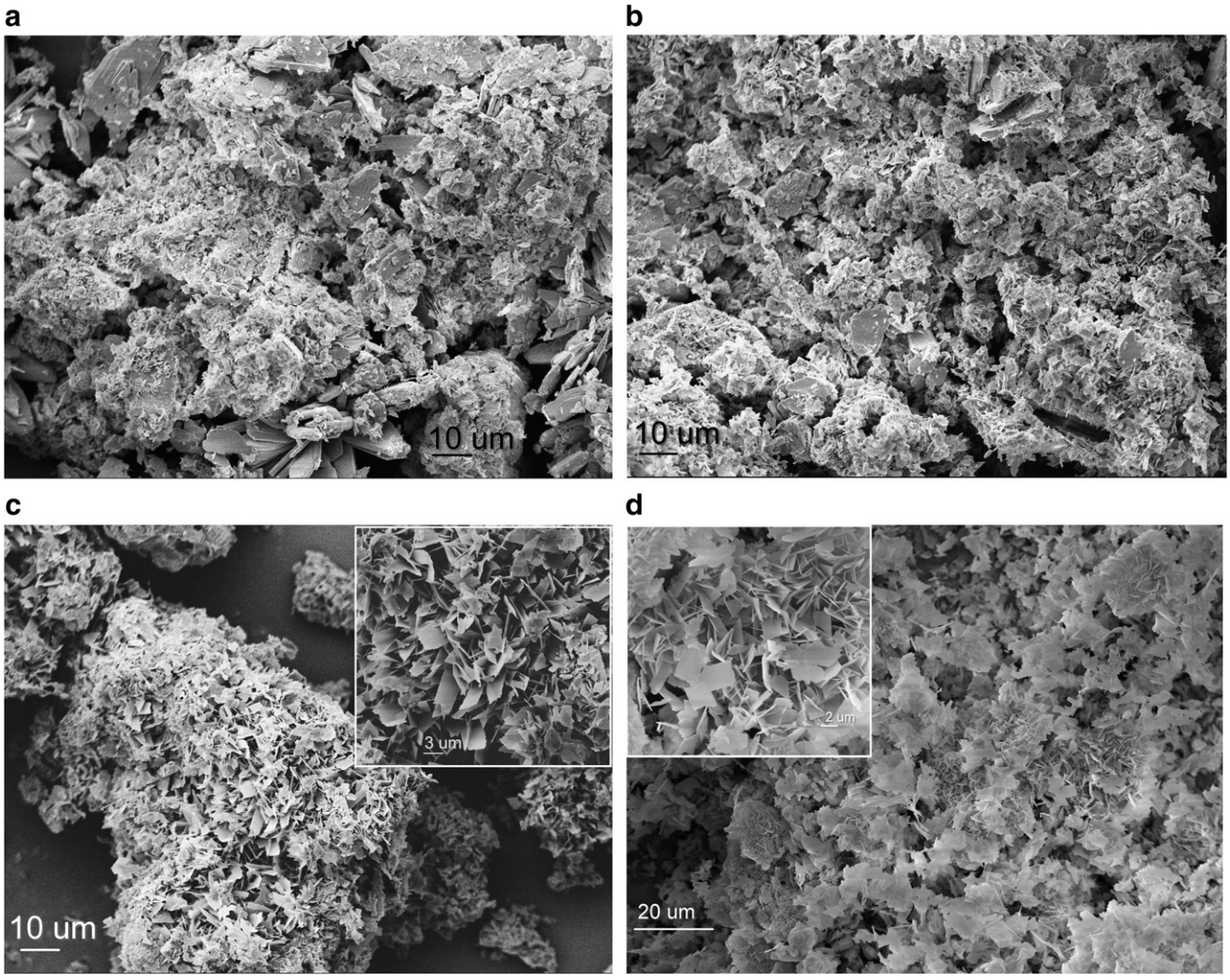

Fig. 4. SEM photomicrographs of samples soaked in DMEM solutions at 36.5 °C; (a) WL — 24 h, (b) WL — 72 h, (c) WL — 1 week, and (d) FP — 1 week.

soluble complexes with several cations, including the most important Ca^{2+}. This would in turn help to reduce the concentration of free Ca^{2+} ions in SBF with respect to an SBF-like solution without Tris [56]. The pH of DMEM solutions (containing the brushite powders) of the current study decreased from 7.4 to around 6.77 by the end of the first 72 h at 36.5 °C, then gradually rose to 6.83 over the following 48 h of immersion. The pH values of the brushite-containing DMEM solutions were found to rise to 7.0 at the end of 7 days at 36.5 °C. This meant that the Hepes-buffered DMEM solutions were not able to maintain the initial pH of 7.4 when they had the slightly acidic brushite powders in them. The SBF-without-Tris solutions of Serro and Saramago [56], on the other hand, aged at 37 °C were found to exhibit pH values increasing from 7.4 to values of 8.5 (while precipitating HA) in the course of 7 days, whereas the same authors reported the corresponding pH variations for Tris-buffered SBF solutions to be below 0.1 within the same timeframe. Does this mean that maintaining the pH value at or not below 7.4 would be essential for producing HA from such solutions?

The morphology of the OCP crystals deposited on titanium, HA or HA-TCP samples [51–55] of previous studies were quite similar to those shown in Fig. 4c and d.

The influence of amino acids present in MEM solutions, in direct comparison to HBSS (Hanks' balanced salt solution) [57], was studied by Hiromoto et al. [58] in terms of the amount of calcium phosphate deposited on titanium coupons immersed in HBSS (Hanks' balanced salt solution) or MEM solutions over 7 days. HBSS solutions do not contain amino acids and any Tris or Hepes buffers [57]. The presence of biomolecules (in the case of MEM solutions) was found to decrease the amount of calcium phosphate deposited [58]. One of the most important contributions of the Hiromoto et al. [58] study had been the quantitative measurement of the Ca, P, C, N, and O amounts of the surface oxide films of titanium specimens immersed in HBSS and MEM solutions by using X-ray photoelectron spectroscopy. Quite small amounts of carbon and nitrogen (and especially nitrogen) detected in the calcium phosphate layers coated on titanium were originating directly from the amino acids present in the MEM solutions. Hiromoto et al.'s [58] study thus provided a strong evidence for the adsorption and incorporation of biomolecules, supplied by the amino acids of MEM solutions, in the newly forming calcium phosphate phases in such biocompatible media.

Although the experimental scope of our study was not wide enough to include the investigation of the influence of biomolecules (and their adsorption) on the brushite-to-OCP transformation occurring in DMEM at 36.5 °C, it shall be not so unsafe to assume the adsorption of biomolecules on our OCP crystals.

The role of bovine serum albumin (BSA) on the crystallization of OCP on type I collagen of bovine origin in metastable supersaturated solutions of pH = 6.5 at 37 °C was studied by Combes et al. [59]. BSA was found to strongly influence the shape of the OCP crystals at the quite high level of 40 g/L, resulting in smaller crystals with curved edges [59]. The amino acids present in our DMEM solutions did not exhibit such an

effect on the crystal morphology of OCP as seen in Fig. 4c and d. The SEM photomicrographs shown in Fig. 4c and d resembled to those supplied by Combes et al. [59] when they synthesized their samples in the absence of BSA.

To summarize, if the pH of a physiologic medium (either Hepes-buffered DMEM or Tris-buffered SCS [51]) is dropping to around 7.2–6.8 during hydrothermal ageing, then such a solution would be prone to nucleate crystals of OCP, rather than those of HA.

DMEM solution was found to have the ability of transforming brushite powders into OCP at 36.5 °C within 1 week; OCP is a biological calcium phosphate phase; OCP is indeed the precursor to HA in enamel and bone formation; therefore, the DMEM solutions can be used to test the so-called bioactivity of brushite powders as confidently as SBF solutions. DMEM solutions, which are commonly used in cell culture [60–62], can be regarded as a feasible alternative to using SBF solutions in the so-called *in vitro* bioactivity testing of synthetic biomaterials.

In regard to follow-up studies, the transformation of brushite powders to OCP at the human body temperature, if performed in a cell culture solution such as DMEM, could also prove to be a viable option to synthesize OCP-based biomaterials in solutions, which can be specifically loaded with bioactive molecules, certain proteins and growth factors.

The current study has also offered a new synthesis route to prepare $CaHPO_4 \cdot 2H_2O$ powders with an unprecedented morphology, i.e., water lily(WL)-like brushite dumbbells. The main advantage of the WL (water lily)-type brushite or WL-type monetite powders will be the following. These powders were produced in simple aqueous systems which did not contain any organic or polymeric substances or surfactants. For example, the very recent use of cetyltrimethylammonium bromide (CTAB, a cationic hydrophobic detergent), by Ruan et al. [63], in preparing monetite powders with a flower-like morphology (i.e., a morphology outside the common flat-plate type observed in brushite synthesis) could only be regarded as an interesting attempt, but the resultant monetite powders must surely be suspected of containing the residues of CTAB used during synthesis. CTAB, for instance, is already known to cause chronic toxicity upon its digestion [64,65]. The total absence of organics in the production does eliminate any toxicity concerns about the possible organic residues (even at the ppm levels) to be present in such calcium phosphate-based bone substitute biomaterials.

A new family of calcium phosphate cements with the final setting product being brushite (in stark contrast to more common hydroxyapatite cements) was developed within the last decade and they were claimed to show increased *in vivo* resorbability [66–68]. The investigation of the hydrothermal transformation behavior of brushite at 36.5 °C in a cell culture medium, such as DMEM, was expected to increase the level of scientific understanding on the bioactivity of brushite-based biomaterials.

5. Conclusions

(1) A new chemical process was suggested for the robust and economical synthesis of brushite ($CaHPO_4 \cdot 2H_2O$) powders with a unique water lily-like morphology. The new process involved the simple stirring of an aqueous suspension of precipitated calcite ($CaCO_3$) powders and dissolved ammonium dihydrogen phosphate ($NH_4H_2PO_4$), the suspension being free of any organic additives, at room temperature (24 ± 1 °C), from 6 to 60 min.

(2) The results showed that it was possible to preserve the water lily-like particle morphology of brushite even though the powders were later converted to monetite ($CaHPO_4$) by heating at 200 °C. Monetite powders with water lily-shaped crystals were produced for the first time.

(3) This study offered a simple procedure for producing octacalcium phosphate ($Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$) powders, upon straightforward immersion of brushite powders in DMEM solutions (pH 7.4) at the human body temperature of 36.5 °C. The highest temperature of processing hereby used in the manufacture of bulk, well-crystallized OCP powders was 36.5 °C.

(4) DMEM solutions were shown to be an alternative to the SBF-based bioactivity testing.

Acknowledgements

Within the course of a senior-year graduation project, Ibrahim Mert had helped Dr. A. C. Tas in three supervised experiments during the synthesis of brushite powders by using the $CaCO_3$–$NH_4H_2PO_4$ route described in this manuscript. Both authors are grateful for the generous help offered by Res. Assist. Muge Yazici of the Department of Genetics and Bioengineering of Yeditepe University in capturing the SEM photomicrographs.

***Notes*:** Certain commercial equipment, instruments, solutions 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] T. Kokubo, J. Non-Cryst. Solids 120 (1990) 138.
[2] D. Bayraktar, A.C. Tas, J. Eur. Ceram. Soc. 19 (1999) 2573.
[3] H.M. Kim, K. Kishimoto, F. Miyaji, T. Kokubo, T. Yao, Y. Suetsugu, J. Tanaka, T. Nakamura, J. Mater. Sci. Mater. Med. 11 (2000) 421.
[4] M. Bohner, J. Lemaitre, Biomaterials 30 (2009) 2175.
[5] T. Kokubo, H. Takadama, Biomaterials 27 (2006) 2907.
[6] F.C.M. Driessens, R.M.H. Verbeeck, Biominerals, CRC Press, Boca Raton, FL, 1990, p. 37.
[7] M. Kumar, H. Dasarathy, C. Riley, J. Biomed. Mater. Res. 45 (1999) 302.
[8] D. Walsh, J. Tanaka, J. Mater. Sci. Mater. M. 12 (2001) 339.
[9] L. Grondahl, F. Cardona, K. Chiem, E. Wentrup-Byrne, T. Bostrom, J. Mater. Sci. Mater. M. 14 (2003) 503.
[10] S.J. Lin, R.Z. LeGeros, J.P. LeGeros, J. Biomed. Mater. Res. 66A (2003) 819.
[11] C.Y. Kim, H.B. Lim, Key Eng. Mater. 254-2 (2004) 305.
[12] H.S. Azevedo, I.B. Leonor, C.M. Alves, R.L. Reis, Mater. Sci. Eng. C 25 (2005) 169.
[13] X. Lu, Y. Leng, Biomaterials 26 (2005) 1097.
[14] D.J.T. Hill-Zainuddin, T.V. Chirila, A.K. Whittaker, A. Kemp, Biomacromolecules 7 (2006) 1758.
[15] J. Pena, I. Barba-Izquierdo, A. Martinez, M. Vallet-Regi, Solid State Sci. 8 (2006) 513.
[16] Y. Honda, S. Kamakura, K. Sasaki, T. Anada, T. Masuda, O. Suzuki, Key Eng. Mater. 330–332 (2007) 479.
[17] R. Gildenhaar, G. Berger, E. Lehmann, C. Knabe, Key Eng. Mater. 361–363 (2008) 331.
[18] F. Yang, J.G.C. Wolke, J.A. Jansen, Chem. Eng. J. 137 (2008) 154.
[19] J.A. Juhasz, S.M. Best, A.D. Auffret, W. Bonfield, J. Mater. Sci. Mater. M. 19 (2008) 1823.
[20] L.P. Xu, E.L. Zhang, K. Yang, J. Mater. Sci. Mater. M. 20 (2009) 859.
[21] A. Rakngarm, Y. Mutoh, Mater. Sci. Eng. C 29 (2009) 275.
[22] A.C. Tas, Int. J. Appl. Ceram. Technol. 4 (2007) 152.
[23] S. Jalota, S.B. Bhaduri, A.C. Tas, Mater. Sci. Eng. C 28 (2008) 129.
[24] L. Tortet, J.R. Gavarri, G. Nihoul, A.J. Dianoux, J. Solid State Chem. 132 (1997) 6.
[25] J. Xu, I.S. Butler, D.F.R. Gilson, Spectrochim. Acta A 55 (1999) 2801.
[26] ICDD PDF: International Centre for Diffraction Data, Powder Diffraction File, Newtown Square, Pennsylvania, USA.
[27] W. Wu, G.H. Nancollas, Langmuir 13 (1997) 861.
[28] O. Suzuki, S. Kamakura, T. Katagiri, M. Nakamura, B. Zhao, Y. Honda, R. Kamijo, Biomaterials 27 (2006) 2671.
[29] R.Z. LeGeros, G. Daculsi, I. Orly, T. Abergas, W. Torres, Scanning Microsc. 3 (1989) 129.
[30] R.Z. LeGeros, Calcif. Tissue Int. 37 (1985) 194.
[31] W.E. Brown, J.P. Smith, J.R. Lehr, A.W. Frazier, Nature 196 (1962) 1048.
[32] W.E. Brown, Clin. Orthop. 44 (1966) 205.
[33] M. Iijima, Monogr. Oral Sci. 18 (2001) 17.
[34] L.J. Shyu, L. Perez, S.L. Zawacki, J.C. Heughebaert, G.H. Nancollas, J. Dent. Res. 62 (1981) 398.
[35] X. Guan, R. Tang, G.H. Nancollas, J. Biomed. Mater. Res. 71A (2004) 488.
[36] H.E. Lundager Madsen, Acta Chem. Scand. A 37 (1983) 25.
[37] H.E. Lundager Madsen, J. Cryst. Growth 310 (2008) 2602.
[38] R.Z. Sabirov, J. Prenen, G. Droogmans, B. Nilius, J. Membrane Biol. 177 (2000) 13.
[39] L. Fulop, G. Szigeti, J. Magyar, N. Szentandrassy, T. Ivanics, Z. Miklos, L. Ligeti, A. Kovacs, G. Szenasi, L. Csernoch, P.P. Nanasi, T. Banyasz, Acta Physiol. Scand. 178 (2003) 11.
[40] J. Pratt, J.D. Cooley, C.W. Purdy, D.C. Straus, Curr. Microbiol. 40 (2000) 306.
[41] T. Nordstrom, L.D. Shrode, O.D. Rotstein, R. Romanek, T. Goto, J.N.M. Heersche, M.F. Manolson, G.F. Brisseau, S. Grinstein, J. Biol. Chem. 272 (1997) 6354.
[42] A.F. Schilling, W. Linhart, S. Filke, M. Gebauer, T. Schinke, J.M. Rueger, M. Amling, Biomaterials 25 (2004) 3963.
[43] A. Brandao-Burch, J.C. Utting, I.R. Orriss, T.R. Arnett, Calcif. Tissue Int. 77 (2005) 167.

[44] K.K. Kaysinger, W.K. Ramp, J. Cell. Biochem. 68 (1998) 83.
[45] L.T. de Jonge, J.J.J.P. van den Beucken, S.C.G. Leeuwenburgh, A.A.J. Hamers, J.G.C. Wolke, J.A. Jansen, Biomaterials 5 (2009) 2773.
[46] M.J. Coelho, A.T. Cabral, M.H. Fernandes, Biomaterials 21 (2000) 1087.
[47] P.A. Price, W.S. Chan, D.M. Jolson, M.K. Williamson, Arterioscl. Throm. Vas. 26 (2006) 1079.
[48] H. Newesely, Monatsh. Chem. 91 (1960) 1020.
[49] Y. Liu, P.R. Cooper, J.E. Barralet, R.M. Shelton, Biomaterials 28 (2007) 1393.
[50] H. Monma, T. Okura, Y. Hara, Y. Moriyoshi, Phosphorus Res. Bull. 23 (2009) 10.
[51] H.B. Wen, J.G.C. Wolke, J.R. de Wijn, Q. Liu, F.Z. Cui, K. de Groot, Biomaterials 18 (1997) 1471.
[52] H.B. Wen, J. Moradian-Oldak, J. Biomed. Mater. Res. 64A (2003) 483.
[53] F. Barrere, P. Layrolle, C.A. van Blitterswijk, K. de Groot, J. Mater. Sci. Mater. M. 12 (2001) 529.
[54] F. Barrere, C.M. van der Valk, R.A.J. Dalmeijer, G. Meijer, C.A. van Blitterswijk, K. de Groot, P. Layrolle, J. Biomed. Mater. Res. 66A (2003) 779.
[55] P. Habibovic, C.M. van der Valk, C.A. van Blitterswijk, G. Meijer, K. de Groot, J. Mater. Sci. Mater. M. 15 (2004) 373.
[56] A.P. Serro, B. Saramago, Biomaterials 24 (2003) 4749.
[57] J.H. Hanks, R.E. Wallace, Proc. Soc. Exp. Biol. Med. 71 (1949) 196.
[58] S. Hiromoto, T. Hanawa, K. Asami, Biomaterials 25 (2004) 979.
[59] C. Combes, C. Rey, M. Freche, J. Mater. Sci. Mater. M. 10 (1999) 153.
[60] J.L. Ong, D.R. Villarreal, R. Cavin, K. Ma, J. Mater. Sci. Mater. M. 12 (2001) 491.
[61] J.E. Gough, I. Notingher, L.L. Hench, J. Biomed. Mater. Res. 68A (2004) 640.
[62] T.J. Webster, J.U. Ejiofor, Biomaterials 25 (2004) 4731.
[63] Q.C. Ruan, Y.C. Zhu, Y. Zeng, H.F. Qian, J.W. Xiao, F.F. Xu, L.L. Zhang, D.H. Zaho, J. Phys. Chem. B 113 (2009) 1100.
[64] B. Isomaa, J. Reuter, B.M. Djupsund, Arch. Toxicol. 35 (1976) 91.
[65] M.J. Heffernan, S.P. Kasturi, S.C. Yang, B. Pulendran, N. Murthy, Biomaterials 30 (2009) 910.
[66] B. Flautre, C. Maynou, J. Lemaitre, P. Van Landuyt, P. Hardouin, J. Biomed. Mater. Res. 63B (2002) 413.
[67] 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.
[68] M. Bohner, U. Gbureck, J.E. Barralet, Biomaterials 26 (2005) 6423.