

Chemical Preparation of Carbonated Calcium Hydroxyapatite Powders at 37°C in Urea-containing Synthetic Body Fluids

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Abstract

An important inorganic phase of synthetic bone applications, calcium hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), was prepared as a single-phase ceramic powder. Carbonated HA powders were formed from calcium nitrate tetrahydrate and diammonium hydrogen phosphate salts dissolved in aqueous 'synthetic body fluid' (SBF) solutions, containing urea (H_2NCONH_2), at 37°C and pH of 7.4, by using a novel chemical precipitation technique. These powders were also found to contain trace amounts of Na and Mg impurities in them, originated from the use of SBF solutions, instead of pure water, during their synthesis. The characterization and chemical analysis of the synthesized powders were performed by powder X-ray diffraction (XRD), Fourier-transformed infra-red spectroscopy (FT-IR), and inductively-coupled plasma atomic emission spectroscopy (ICP-AES). © 1999 Elsevier Science Ltd. All rights reserved.

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1 Introduction

Calcium hydroxyapatite (HA: $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), the main inorganic component of the hard tissues in bones, is a member of 'apatite' family of compounds. Biological apatites, which comprise the mineral phases of calcified tissues (enamel, dentin, and bone), slightly differ from pure HA in stoichiometry, composition and crystallinity, and in other physical and mechanical properties. They were usually observed¹ to be carbonate-substituted and calcium-deficient.

Synthetic body fluids, (SBF), prepared in accord with the chemical analysis of human body fluid,

with ion concentrations nearly equal to those of the inorganic constituents of human blood plasma, were first used by Kokubo *et al.*,² to prove the similarity between *in vitro* and *in vivo* behavior of certain glass-ceramic compositions. In these studies, the glass-ceramic samples were soaked in SBF solutions, and their surfaces were observed to be coated with a poorly crystallized calcium deficient and carbonate containing apatite, which was similar to bone apatite.³

HA powders have generally been synthesized from aqueous solutions for use in bioceramic applications. It is known⁴ that calcium hydroxyapatite is the least soluble and the most stable compound of calcium phosphate phases in aqueous solutions at pH values higher than 4.2. However, HA powders synthesized in highly alkaline media^{5–10} were recognized by their relatively high thermal stability and phase purity even after high temperature (1100–1300°C) sintering. Chemical synthesis of HA powders in neutral and/or slightly acidic aqueous media is known to be a more complicated and difficult task.^{7,11} This study¹² will focus on the determination of the experimental parameters of HA synthesis at the physiological pH (7.4) and temperature (37°C), in SBF solutions rich in urea.

K. de Groot *et al.*,⁸ studied the high-temperature characteristics of synthetic HA and reported that the synthetic HA with a Ca/P ratio near to 1.67 was only stable below 1200°C, when sintered in a dry or wet air atmosphere. It was claimed that beyond 1200°C, HA loses its OH groups gradually and transforms into oxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6\text{O}$). When heated to 1450°C, oxyapatite was found⁸ to dissociate into α -TCP, $\text{Ca}_2\text{P}_2\text{O}_7$ and $\text{Ca}_4\text{P}_2\text{O}_9$. On the other hand, Tas *et al.*,¹⁰ recently reported that pure HA powders prepared in pure water may withstand, without decomposition, temperatures as high as 1300°C when heated in a dry air atmosphere for 6 h.

The surface properties and synthesis conditions of calcium hydroxyapatite, prepared by using pure water in a highly alkaline medium, has previously

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been examined in different buffers^{4,6,13} including the SBF; however, there is no published work, to our knowledge, on the 'precipitation and formation' of calcium hydroxyapatite powders in SBF at 37°C. The study presented here, thus, becomes the first systematic step taken in this specific field of 'biomimetic' HA powder synthesis technology.

2 Experimental Procedure

2.1 Preparation of synthetic body fluids (SBF)

SBF is known to be a metastable buffer solution,³ and even a small, undesired variance in both of the preparation steps and the storage temperatures, may drastically affect the phase purity and high-temperature stability of the produced HA powders, as well as the kinetics of the precipitation processes.

Merck (Darmstadt, Germany)-grade NaCl (99.5%), NaHCO₃ (99.5%), KCl (99.0%), Na₂HPO₄·2H₂O (99.5%), MgCl₂·6H₂O (98%), Na₂SO₄, (CH₂OH)₃CNH₂ (99.2%), CaCl₂·2H₂O (99%) and HCl (37 vol%, Carlo-Erba, Italy) were used in the preparation of the synthetic body fluids. SBF solutions were prepared¹² by dissolving appropriate quantities of the above chemicals in de-ionized water. Reagents were added, one by one after each reagent was completely dissolved in 700 ml of water, in the order given in Table 1.

A total of 40 ml of 1 M HCl solution was consumed for pH adjustments during the preparation of SBF solutions. The 15 ml aliquot of this amount was added just before the addition of the 6th reagent, i.e., CaCl₂·2H₂O. The second portion of the HCl solution was used in the remainder of the titration process. Following the addition of the 8th reagent (tris(hydroxymethyl)aminomethane), solution temperature was raised from the ambient to 37°C. This solution was then titrated with 1 M HCl to the pH of 7.4, at 37°C. During the titration process, the solution was also continuously diluted with consecutive additions of de-ionized water to make the final volume equal to 1 l. It was observed

in this study that the prepared SBF solutions can be stored at 5°C for a month without degradation.

The ion concentrations of the SBF solutions used in this study were matched more closely with those of 'human plasma' than the ones given by Kokubo and his co-workers.^{2,3} The most significant differences of our study, as compared to the previous workers, in terms of 'SBF ion concentrations' may be summarized as follows:

- the nominal, initial HCO₃⁻ ion concentration of SBF was increased from 4.2 to 27.0 mM which, thus, acquired exactly the same concentration figure of human plasma,
- Cl⁻ ion concentration was decreased from 147.8 mM to 125 mM whereas the Cl⁻ ion concentration of human plasma is 103 mM.

The differences between the ion concentrations of our SBF solutions and those of human plasma are compared in Table 2.

2.2 Synthesis of HA powders in SBF solutions

Ca(NO₃)₂·4H₂O (Riedel de-Haen, 99%, Germany) and (NH₄)₂HPO₄ (Merck, 99%, Germany) stock solutions of proper concentration were used in the precipitation experiments as the calcium and phosphate ion sources. Urea solutions (9.5 M) (by dissolving the urea powder in SBF) were prepared and aged at 85°C overnight, to ensure its decomposition. The pH values of such 'aged urea' solutions were measured as 9.3, prior to their use, at 37°C. Small aliquots of NH₄OH (12 vol%) solutions were used for pH adjustments (to keep the solution pH constant at 7.4), when necessary.

Two different precipitation schemes were used during our HA powder synthesis experiments. These two routes were given in the flowcharts of Figs. 1 and 2. The first scheme of synthesis may be called the 'initial addition-type', since it involves the addition of the inorganics at the beginning. The second scheme of synthesis used was called the 'continuous addition-type' due to the continuous addition of the phosphate salt into the nitrate by a peristaltic pump.

Table 1. Chemical composition of SBF solutions^a

Order	Reagent	Amount (gpl)
1	NaCl	6.547
2	NaHCO ₃	2.268
3	KCl	0.373
4	Na ₂ HPO ₄ ·2H ₂ O	0.178
5	MgCl ₂ ·6H ₂ O	0.305
6	CaCl ₂ ·2H ₂ O	0.368
7	Na ₂ SO ₄	0.071
8	(CH ₂ OH) ₃ CNH ₂	6.057

^aPatent pending, Turkish Patent Institute, Turkey, Appl. No. 99/0037, 11 January 1999.

Table 2. Ion concentrations of SBF solutions and 'human plasma'

Ion	Kokubo et al. ^{2,3} (mM)	Present work (mM)	Human plasma (mM)
Na ⁺	142.0	142.0	142.0
Cl ⁻	147.8	125.0	103.0
HCO ₃ ⁻	4.2	27.0	27.0
K ⁺	5.0	5.0	5.0
Mg ²⁺	1.5	1.5	1.5
Ca ²⁺	2.5	2.5	2.5
HPO ₄ ²⁻	1.0	1.0	1.0
SO ₄ ²⁻	0.5	0.5	0.5

In the 'initial addition-type' HA synthesis scheme, 204 ml of SBF and 20 ml of urea (9.5 M, aged) solution were first mixed by an immersion stirrer in a 300 ml beaker. Following the addition of this much urea into the SBF solution, the final HCO_3^- concentration in this beaker was calculated to rise to 0.75 M from the initial value of 27 mM (of pure SBF). This beaker was then placed in a water bath kept at 37 °C. The pH value of this solution was initially 8.2 at 37 °C. 1.583 g of $(\text{NH}_4)_2\text{HPO}_4$ and 5.1 g of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ powders were weighed and added to the urea-SBF solution at once. Following the immediate formation of fine precipitates, the solution was stirred at 37 °C for 90 min. NH_4OH (12 vol%) additions were made in an intermittent but continuous manner (by a pipette) to fix the solution pH in the vicinity of 7.4. The pH of the solution just after the addition of the powders was measured as 6.9. During the whole experiment, only a total of 2 ml ammonia solution addition was needed to maintain the pH at the physiological value. Following the 90 min of aging of the precipitates in the water bath at 37 °C, ultrasonic irradiation was applied, via an ultrasonic disruptor (Misonix, XL-2015, USA), until the temperature of the solution increased to 45–50 °C, and the solution was then left overnight at 37 °C in a microprocessor-controlled oven. The precipitates were later separated from the mother liquor by filtration, and washed with de-ionized

water by 4 times. The filtrates were dried in air overnight at 80 °C. The dried powders were calcined in a stagnant air atmosphere over the temperature range of 1100–1600 °C for varying times of 6–17 h.

In the 'continuous addition-type' HA synthesis route, 148.9 ml of 0.218 M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ solution was placed in a 400 ml beaker, inserted in a water bath kept at 37 °C. Urea (23 ml) (9.5 M, aged) was then added to this beaker and stirred. 211.7 ml of 0.085 M $(\text{NH}_4)_2\text{HPO}_4$ solution was added in a drop-wise fashion via a peristaltic pump at the rate of 4.5 ml min⁻¹. At the beginning of the experiment, the pH value was 7.4 and started to decrease with the drop-wise addition of the phosphate solution. NH_4OH (12 vol%) additions were made in an intermittent but continuous manner (by a pipette) to fix the solution pH in the vicinity of 7.4. During the whole experiment, only a total of 4.5 ml of ammonia solution addition was needed to maintain the pH at the physiological value. The rest of the powder processing steps were similar to those of the 'initial addition-type.'

For comparison purposes, the 'continuous addition-type' HA synthesis experiments were repeated, under strictly the same conditions, in 'pure SBF' solutions without any dissolved urea.

2.3 Powder characterization

The phase purity and the levels of crystallinity of the calcined HA powders were studied by an X-ray

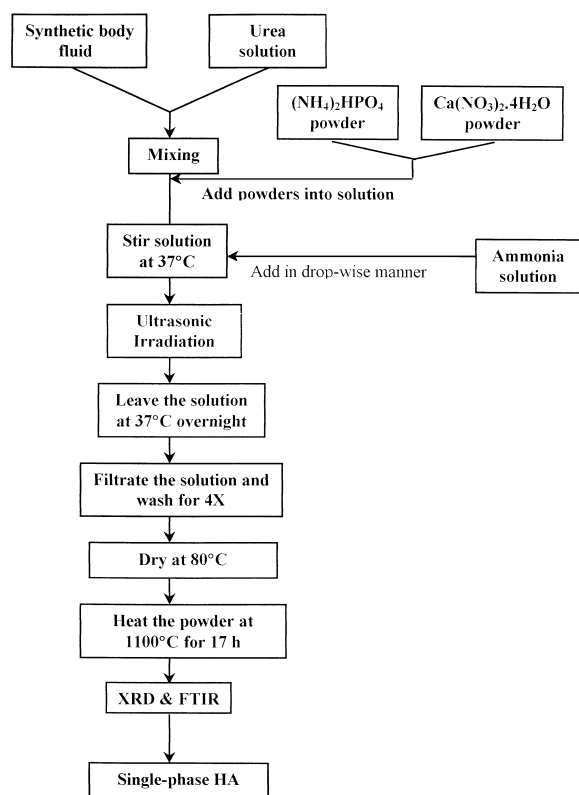


Fig. 1. Process flowchart for HA synthesis by the 'initial addition' scheme.

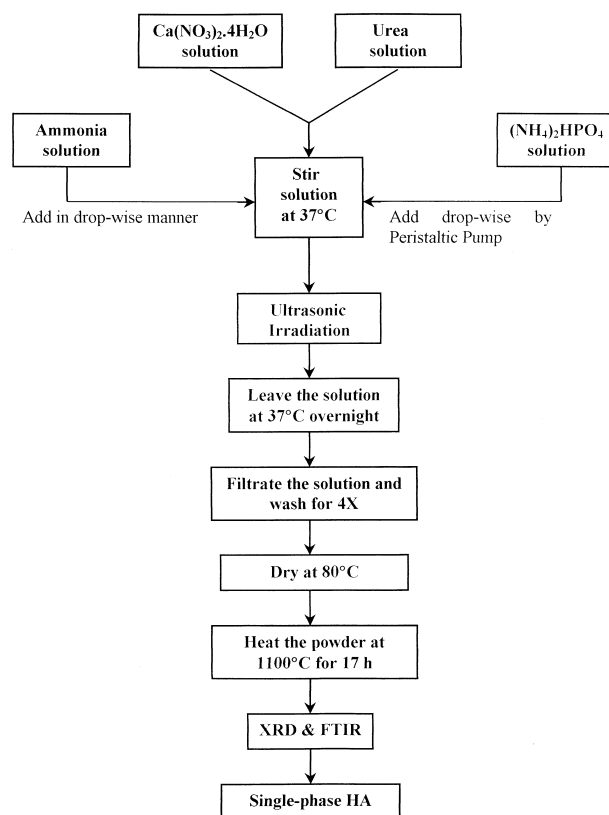


Fig. 2. Process flowchart for HA synthesis by the 'continuous addition' scheme.

diffractometer (XRD) (Model: D-Max/B, Rigaku Co., Tokyo, Japan) at the typical step size of $0.05^\circ 2\theta$ and a count time of 1 s. A Cu- K_α tube operated at 40 kV and 20 mA was used for the generation of X-rays. All of the collected X-ray spectra were corrected for the shifts in the d-spacings by using pure silicon (Starck Inc., Germany) as an external standard. A least-squares unit cell refinement algorithm (Appleman & Evans)¹⁴ was used for the precise determination of crystallographic parameters of the synthesized powders. The further details of XRD analysis were described elsewhere.¹⁵

Fourier-transformed infra-red spectroscopy (FT-IR) (Model: DX-510, Nicolet Co., WI, USA) was used in the wave number range of $4000\text{--}400\text{ cm}^{-1}$. Experimental spectra of solid samples were obtained by preparing KBr pellets with a 3:100 'sample-to-KBr' ratio.

2.4 Chemical analysis

Inductively-coupled plasma atomic emission spectroscopy (ICP-AES) (Model Plasma-1000, Perkin-Elmer Co., London, UK) was used for the accurate chemical analysis of Na and Mg ions in the SBF-synthesized HA powders (after being dissolved in acid solutions) which were believed to originate from the use of 'synthetic body fluids' during our synthesis experiments.

3 Results and Discussion

Biological apatites mainly differ from the synthetically produced calcium hydroxyapatites in terms of their carbonate ion content (i.e. the carbonate ion levels being incomparably lower in the latter). Body fluids may be considered as a biomimetic and logical source for increasing the carbonate contents of synthetically prepared HA bioceramics. However, the significantly low levels of HCO_3^- (i.e. 27 mM) in human plasma or SBF solutions makes it impractical for their economically workable use within the shorter 'aging' times allowed in powder synthesis technology, as compared to the aging of natural bones of offsprings in the human body for a typical period of '9 months + 10 days'. The pursuit for the manufacture of a genuinely 'carbonated' HA powder mainly originated from a clinical demand for the synthesis of an HA powder with improved *in vivo* biocompatibility. We have hereby tried the taking of a first systematic step into the comparison of the carbonate ion incorporation into the resultant hydroxyapatite precipitates; (i) grown in pure SBF, and (ii) those grown in urea-containing SBF solutions, under the physiological conditions of 37°C and pH of 7.4.

The as-filtered/dried calcium phosphate precipitates formed at 37°C and pH of 7.4 via 'pure SBF' (i.e. without urea) solutions were found to be amorphous. Upon calcination of these amorphous powders, in a stagnant air atmosphere, at 1100°C for 6 h, they completely transformed into crystalline, single-phase HA. However, it has long been a well-confirmed experimental fact^{6,8,10} that the synthetically prepared (via aqueous chemical precipitation techniques) HA powders usually decomposed (partially) into tri-calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$), calcium pyrophosphate ($\text{Ca}_2\text{P}_2\text{O}_7$), tetra calcium phosphate ($\text{Ca}_4\text{P}_2\text{O}_9$), and/or into oxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6\text{O}$) when heated above 1200°C in air.

The HA precipitates of this study formed in 'pure SBF' solutions, uncommonly, had an excellent thermal stability even when heated at 1600°C for 6 h in an air atmosphere. Figure 3 shows the XRD traces of pure SBF-synthesized HA precipitates heated at different temperatures, over the range of $1300\text{--}1600^\circ\text{C}$, for 6 h. The XRD spectra of all samples closely matched with the ICDD PDF No: 9-432 for calcium hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$).

These powders also contained trace amounts of other inorganic ions, provided by SBF. ICP analysis performed on representative 1200°C calcined samples indicated that these HA powders had 0.16% Mg and 0.08% Na. Na^+ and Mg^{2+} ions, originated from the use of SBF solutions during precipitation, were believed to incorporate themselves in the crystal structure of calcium hydroxyapatite. The lattice parameters of these 'pure SBF'-grown powders were measured as $a = 9.4168$

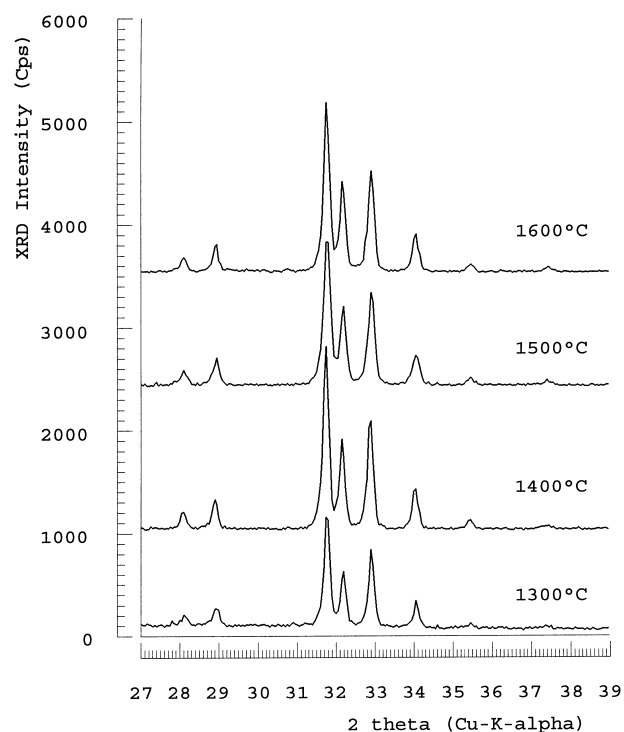


Fig. 3. XRD traces of HA precipitates formed in pure SBF, after heating at different temperatures ($1300\text{--}1600^\circ\text{C}$, 6 h).

and $c=6.8760$ Å. The hexagonal unit cell (of the space group $P6_3/m$) volume was equal to 527.95 Å³. The CO_3^{2-} ions are known¹ to substitute, in part, the OH^- sites, and again, in part the PO_4^{3-} groups of the HA structure. On the other hand, Mg^{2+} ions were claimed¹ to replace the Ca^{2+} sites of the HA structure. We also believe that the Na^+ ions might have placed themselves nearby the OH^- sites (close to the P-bonded oxygens), and hence, caused a reduction in the extent of 'de-hydroxylation' at the significantly high calcination temperatures of this study.

The FT-IR spectra of SBF-synthesized HA powders are given in Fig. 4. The top spectrum of this figure belonged to 'pure SBF' grown HA precipitates, after 6 h calcination at 700°C in air. The CO_3^{2-} ion peaks, at 2368 – 2361 (combination, $\nu_2 + \nu_3$), 1467 – 1412 and 878 cm^{-1} , were visible. As the calcination temperature increased, the peaks of the CO_3^{2-} totally disappeared. The OH^- stretching vibration was observed at 3571 cm^{-1} and the OH^- bending vibration was recorded at 635 cm^{-1} . The PO_4 bands¹⁶ were detected at 470 (ν_2), 570 and 603 (ν_4), 962 (ν_1), 1045 and 1096 (ν_3) cm^{-1} . The bottom FT-IR spectrum in Fig. 4 was obtained from the powder synthesis experiments which used the 'urea-containing' SBF solutions. The significant presence (in a qualitative manner) of carbonate

ions in HA structure was again clearly apparent. A further set of decisive carbon analysis would, however, be required for a quantitative determination of the amounts of CO_3^{2-} ions present in such samples.

An interesting difference was also observed between the 'initial' and 'continuous' addition-type HA synthesis experiments (in 'urea-containing SBF' solutions at 37°C) of this study. The 'initial addition'-type HA powders had the lattice parameters of $a=9.4261$ and $c=6.8832$ Å with a unit cell volume of 529.64 Å³. These parameters were considerably larger than those of the HA samples prepared in 'pure SBF' solutions. On the other hand, the 'continuous addition'-type HA powders had the lattice parameters of $a=9.4175$ and $c=6.8753$ Å, with the corresponding unit cell volume of 528.07 Å³. It was interesting to note here that the 'pure SBF' (i.e. no urea) and 'continuous addition'-type HA samples did almost yield the same lattice parameters, which are the well-known¹ lattice parameters for the synthetically produced HA samples. The comparative XRD spectra of both (initial and continuous) HA samples (after calcination at 1100°C for 17 h) were given in Fig. 5. It was also notable to see that the 'continuous addition' samples had the peak of free CaO (at $37.36^\circ 2\theta$), while the 'initial addition' samples did not. This observation may also help

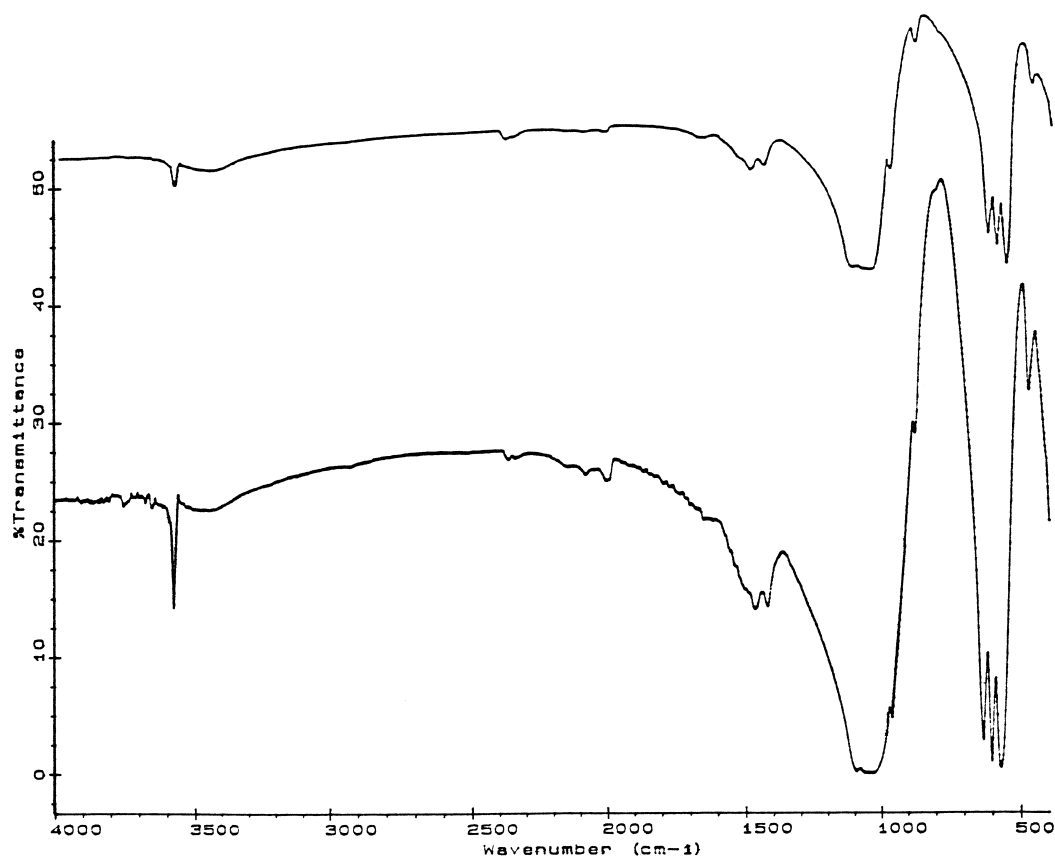


Fig. 4. FT-IR spectra of HA powders prepared in SBF solutions (top: in pure SBF; bottom: in urea-containing SBF, samples heated at 700°C for 6 h).

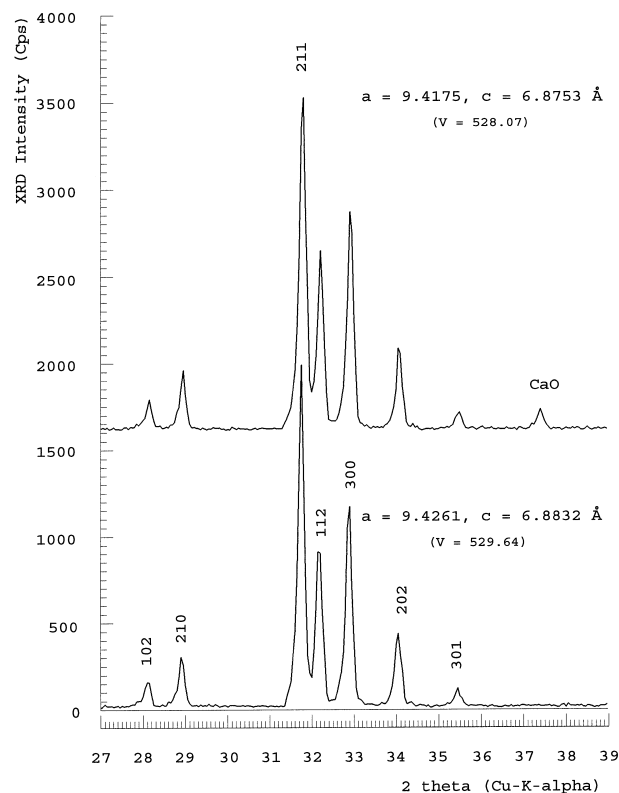


Fig. 5. Comparative XRD spectra of both 'initial' and 'continuous' HA samples (after calcination at 1100 °C for 17 h).

on the speculation that the showing up of 'free' CaO in the XRD spectra might well be a sign of a small but certain degree of deficiency prevailing over the Ca^{2+} sites of the HA structure.

In the 'continuous addition'-type synthesis runs, the Ca/P ratio in the precipitation beakers first started from a very high value, and then gradually converged, in synthesis time, towards that of the proper HA stoichiometry (i.e. 1.67). In the 'initial addition'-type experiments, on the other hand, the Ca/P ratio in the precipitation beakers were 'fixed' at the HA stoichiometry at the very beginning of the process. The observation of excess CaO in the XRD traces of continuous addition-type runs would then not be so surprising from this point of view.

The synthesis of HA powders via 'pure' (i.e. de-ionized water) aqueous solutions by using the starting chemicals of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and $(\text{NH}_4)_2\text{HPO}_4$ requires high pH values in excess of 10.⁶⁻¹¹ On the other hand, in case of using SBF, the pH value needed for HA synthesis decreases^{4,12} considerably. In combination with the initial presence of spontaneously formed seed crystals¹² in precipitation solutions, the high ionic strength of the precipitation medium (provided by SBF) played an important role in inducing the formation of pure calcium hydroxyapatite phase that would otherwise, normally, not nucleate at such a low pH value of 7.4.

4 Summary

Chemically homogeneous, single-phase calcium hydroxyapatite (HA) powders have successfully been synthesized from a novel synthetic body fluid solution, at the physiological and biomimetic conditions of pH of 7.4 and 37 °C, containing dissolved calcium nitrate and di-ammonium hydrogen phosphate salts in appropriate amounts, in the presence of urea, by a chemical precipitation technique. The produced HA powders were shown to be 'carbonated' and to have unprecedented phase stability even when heated at 1600 °C for 6 h, in a stagnant air atmosphere.

Such HA powders were also shown to have small amounts of other inorganic ions, provided and incorporated into the HA structure by the SBF solutions used during synthesis. ICP analysis performed on 1200 °C-calcined powders indicated that the biomimetic HA powders of this study had 0.16% Mg and 0.08% Na. These ions, originated from the SBF solutions, were conjectured to place themselves within the HA crystal structure.

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