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Submicron spheres of amorphous calcium phosphate forming in a stirred SBF solution at 55 °C

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ABSTRACT

X-ray-amorphous calcium phosphate (ACP) spheres were synthesized in a simulated/synthetic body fluid (SBF) solution heated to 55 to 70 °C under constant stirring at 850 rpm. The specific SBF solution (*Lac*–SBF) was buffered by using Na-L-lactate and lactic acid, and did not contain any Tris or Hepes. The *Lac*–SBF solution of this study flawlessly matched the concentrations of the inorganic electrolyte ions of the human blood plasma. The monodisperse ACP spheres synthesized at 55 °C were 245 nm in diameter when the *Lac*–SBF solution contained 67 mg/L gelatin. Samples were characterized by powder X-ray diffraction, Fourier-transform infrared spectroscopy, scanning electron microscopy and inductively-coupled atomic emission spectroscopy.

1. Introduction

The inorganic electrolyte portion of human blood plasma [1], sometimes called extracellular fluid (ECF), is comprised of a balanced mixture of Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}, HPO_4^{2-}, HCO_3^{-}, Cl^{-} and SO_4^{2-} ions in water. SBF (simulated/synthetic body fluid) solutions were developed for the in vitro testing of synthetic biomaterials at 37 °C in aqueous media mimicking the inorganic ion concentrations of blood plasma [2–6]. This article reports the discovery of an unknown ability of stirred SBF solutions (with or without gelatin) when they are simply heated to, for instance, 55 °C. The previous literature lacks any attempts to heat an SBF solution to a temperature above 37 °C.

As shown in Table 1, only two of the known SBF solutions are able to match the ion concentrations of blood plasma: (*i*) 50 mM Hepes (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*, $C_8H_{18}N_2O_4S$)-buffered SBF [7,8] and (*ii*) 22 mM Na-L-lactate ($NaCH_3CH(OH)COO$)-buffered SBF [9,10]. The highly popular conventional SBF solution [4], on the other hand, contains 50 mM Tris (*tris(hydroxymethyl)aminomethane*, $(HOCH_2)_3CNH_2$) and presents a significant difficulty in matching the HCO_3^{-} and Cl^{-} concentrations (Table 1) of human blood plasma.

The lactated Ringer's solution (LRS or RLS, Ringer's lactated solution) is widely used in hospitals, with no adverse effects reported in patients, for intravenous or subcutaneous administration [11]. LRS contains 28 mM Na-L-lactate [11,12]. Since Tris and Hepes (of Table 1) are not present in any bio-fluid, an SBF solution buffered at pH 7.4 by using 22 mM Na-L-lactate and quite small aliquots of lactic acid was used in this study.

The literature does not have any reports on SBF solutions heated at temperatures above 37 °C and this study becomes the first one to do so.

2. Experimental procedure

2.1. Materials and solution preparation

Calcium chloride dihydrate (>99.5%, $CaCl_2 \cdot 2H_2O$, Fisher Scientific, Catalog No: C79), magnesium chloride hexahydrate (>99.5%, $MgCl_2 \cdot 6H_2O$, Fisher, No: AC19753), potassium chloride (>99.5%, KCl, Sigma, No: P3911), sodium hydrogen carbonate (>99.9%, $NaHCO_3$, Merck, No: 106329), sodium chloride (>99.8%, NaCl, Merck, No: 106404), sodium sulfate (>99.5%, Na_2SO_4, Acros, No: 21875), disodium hydrogen phosphate (>99.5%, Na_2HPO_4, Fisher, No: S374), sodium L-lactate (>99.5%, $NaCH_3CH(OH)COO$, Sigma, No: L7022) and 1 M lactic acid solution (>99%, $C_3H_6O_3$, Fluka, No: 35202) were used in solution preparation. De-ionized water (18.2 MΩ) was used in all experiments.

One liter of the Na-L-lactate/lactic acid-buffered SBF solution (i.e., *Lac*–SBF) was prepared by adding the indicated amounts of chemicals in Table 2, in the order given, to 997 mL of pre-boiled de-ionized water. Small aliquots of 1 M lactic acid are added dropwise with a 1 mL pipette, to lower the pH to the physiological value of 7.4, at the final step of solution preparation. As-prepared *Lac*–SBF solutions had a pH value (7.40 ± 0.01 at both room temperatures, 22 ± 1 °C, and 36.5 °C) similar to that of blood plasma. Calcium phosphate syntheses were performed in heat-sterilized (130 °C, 8 h) and unused glass beakers. Inorganic ion concentrations shown in Table 2 match those of

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0022-3093/

Table 1
Compositions of blood plasma versus SBF solutions.

Ion	Blood plasma [1] mM	Convent–SBF [4] mM	Hepes–SBF [7,8] mM	*Lac*–SBF [9,10] mM
Na^+	142.0	142.0	142.0	142.0
K^+	5.0	5.0	5.0	5.0
Mg^{2+}	1.5	1.5	1.5	1.5
HCO_3^-	27.0	4.2	27.0	27.0
Cl^-	103.0	148.8	103.0	103.0
Ca^{2+}	2.5	2.5	2.5	2.5
HPO_4^{2-}	1.0	1.0	1.0	1.0
SO_4^{2-}	0.5	0.5	0.5	0.5
		50 mM Tris	50 mM Hepes	22 mM Na-L-lactate

the human blood plasma. Solutions were stored in sealed glass bottles in a refrigerator (+4 °C) when not in use.

2.2. Synthesis

One liter of the *Lac*–SBF solution of Table 2 was placed in a clean glass beaker. The Parafilm®-covered beaker having *Lac*–SBF, containing a glass thermometer, was placed on a hot-plate and heated to 55°, 65° or 70 ± 1 °C, with constant stirring at 850 rpm using a magnetic stir bar (1 cm-thick and 5 cm-long Teflon®-coated bar used in a 1500 mL glass beaker). The total heating time at the target temperature (55°, 65° or 70 °C) was kept constant at 15 min, counted from the start of visible precipitation. Solutions started to display a bluish tint (i.e., the onset of colloidal particle formation) when the temperature reached around 40 °C. Particles aged at 55°, 65° or 70 °C for 15 min were separated from their mother solution by centrifugation at 10,000 rpm or by filtering through a 0.22 μm filter membrane and then washed with 1 L of deionized water, followed by drying at room temperature, for 36 h, in an air atmosphere.

Bovine gelatin (Mallinckrodt Chemicals, Type B, Cat. No: H219-59), at the constant amount of 67 mg/L, was dissolved in freshly prepared *Lac*–SBF solutions, prior to synthesis runs, in a few experiments.

2.3. Sample characterization

Prior to powder X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR) analyses, the dried samples were manually ground in an agate mortar by using an agate pestle. XRD runs were performed (Advance D8, Bruker, Karlsruhe, Germany) in the step scan mode, with a step size of 0.02° and preset time of 3 s. The powder X-ray diffractometer was equipped with a monochromatic Cu tube and operated at 40 kV and 40 mA. XRD powder samples were prepared by gently packing the powders into single-crystal quartz sample holders with a cavity of around 1 mm-deep.

FTIR samples were mixed with KBr powders at the ratio of 1 mg sample-to-250 mg KBr in an agate mortar using an agate pestle. FTIR pellets with a diameter of 10 mm were pressed at a load of 10 t applied for 1 min. FTIR data were collected (Spectrum One, PerkinElmer, Waltham, MA) using 128 scans at 2 cm^{-1} resolution.

Samples for scanning electron microscopy (SEM, Zeiss-Neon 40 EsB, Oberkochen, Germany) were not ground and small portions of samples embedded on conducting carbon tapes were sputter-coated with a thin (approx. 5 nm thick) layer of gold prior to imaging at 10 kV at a working distance of 7 to 8 mm. Quantitative calcium, magnesium and phosphorus analyses of powder samples were performed by using inductively-coupled plasma atomic emission spectroscopy (ICP-AES, Model 61E, Thermo Electron, Madison, WI). For the ICP-AES analyses, 70 mg portions of powder samples were dissolved in 5 mL of concentrated HNO_3 solution. A combustion analyzer (EMIA-8110, Horiba, Edison, NJ) was used to determine the carbon contents of samples. Elemental analyses were repeated thrice.

Table 2
Preparation of 1 L of *Lac*–SBF [9,10,13] solution.

Chemical	Amount (g/L)	Ion	Concentration (mM)
NaCl	5.2599	Na^+	142.0
$NaHCO_3$	2.2682	Mg^{2+}	1.5
KCl	0.3728	K^+	5.0
$MgCl_2 \cdot 6H_2O$	0.3049	Ca^{2+}	2.5
Na_2SO_4	0.0710	HPO_4^{2-}	1.0
$CaCl_2 \cdot 2H_2O$	0.3675	HCO_3^-	27.0
Na_2HPO_4	0.1419	Cl^-	103.0
Na-lactate	2.4653	SO_4^{2-}	0.5
1 M lactic acid	1.6 mL	Ca/P molar ratio	2.5

3. Results

The particles synthesized by aging the *Lac*–SBF solutions at 55°, 65° or 70 °C for 15 min were all found to be X-ray-amorphous (Fig. 1a). The XRD data did not contain any reflections of apatitic calcium phosphate. The characteristic FTIR spectra of these powders are shown in Fig. 1b. The samples were carbonated as indicated by the bands observed at 1490–1425 and 870 cm^{-1}. The bands for the P–O vibrations of the orthophosphate group are similar to those previously observed for amorphous calcium phosphates [14], and are seen at 1045, 952

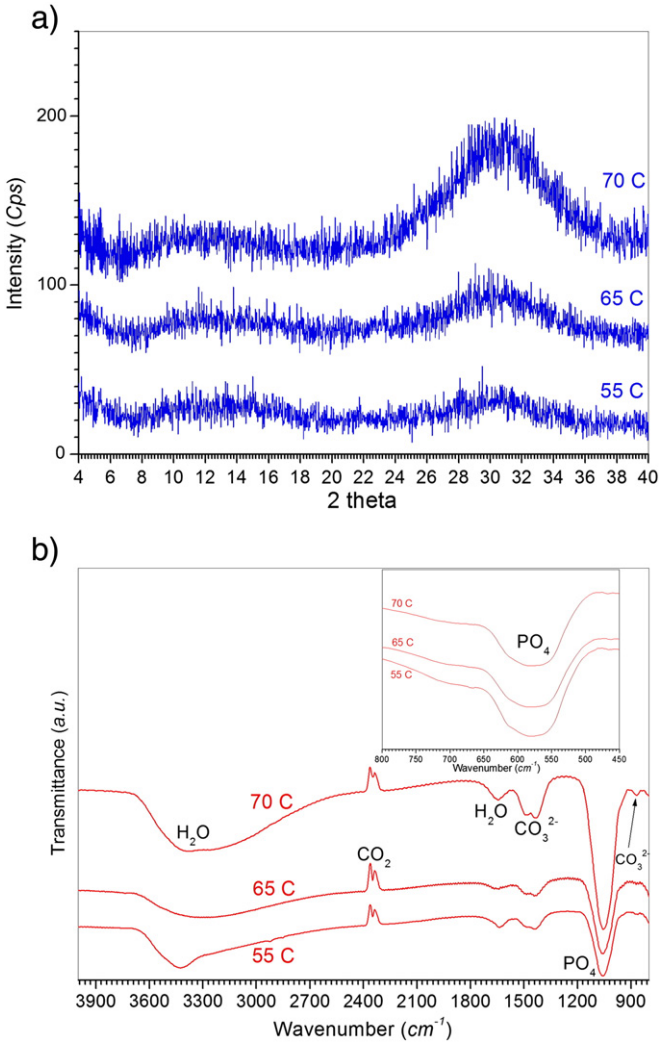

Fig. 1. (a) XRD and (b) FTIR spectra of ACP (amorphous calcium phosphate) samples synthesized at different temperatures in *Lac*–SBF.

and 569 cm^{-1}. No lactate groups were observed in the FTIR data. Further experimental confirmation of the X-ray-amorphous nature of these samples came from the single broad band observed at 569 cm^{-1} [15,16] and this band splits into two even for poorly crystallized (i.e., cryptocrystalline) apatitic calcium phosphate samples. The strong bands of water vibrations at 3350 and 1650 cm^{-1} prove the hydrated nature of these samples. Therefore, the *Lac*–SBF solution of this study heated to 55–70 °C autogenously produced ACP (amorphous calcium phosphate) powders. This ability of SBF solutions was not reported before since they were always used at 37 °C.

Lac–SBF solutions heated at 65° and 70 °C for 15 min produced agglomerated spherical particles close to 1 or 0.6 μm in diameter, respectively, as shown in the SEM photomicrographs of Fig. 2a and b. These samples also contained 4 to 15 μm-long calcium phosphate chunks, which consisted of densely agglomerated particles. The chunks with smooth surfaces (Fig. 2b) have probably grown while in close contact with the surfaces of the glass beaker. Such microscopically smooth surfaces are usually observed in amorphous substances.

Lac–SBF solutions heated at 55 °C for 15 min following the observation of the bluish tint, on the other hand, gave much smaller (approximately 0.3 μm) spherical particles (Fig. 2c). The average particle diameters reported above were determined by using the Heyn's lineal intercept method (in accord with the ASTM standard E112-10) [17] based on the results of 60 measurements on each photomicrograph of Fig. 2.

The change in solution pH, as a function of solution temperature and time, for a typical 55 °C-run is depicted in Fig. 2d. The two vertical arrows in Fig. 2d indicate the start and end of the 15 min synthesis period. The initial rise in pH from 7.4 to 7.5 is caused by the evolution of CO_2 (g) according to Eq. (1a), whereas the decrease in pH from the 13th to 26th minute (Fig. 2d) can be explained by Eq. (1b):

$$H^+(aq) + CO_3^{2-}(aq) = OH^-(aq) + CO_2(g) \quad (1a)$$

$$HCO_3^-(aq) = H^+(aq) + CO_3^{2-}(aq) \quad (1b)$$

The addition of 67 mg/L gelatin to the freshly prepared *Lac*–SBF solutions and the consequent heating of those at 55 °C for 15 min resulted in the production of monodisperse, submicron X-ray-amorphous calcium phosphate spheres (Fig. 3a and b). XRD and FTIR data of these spheres were indistinguishable from those given in Fig. 1a and b for the 55 °C samples. The average diameter of such monodisperse spheres were 245 ± 20 nm. These constitute the very first amorphous calcium phosphate (ACP) monodisperse and submicron spheres synthesized in an SBF solution. This is the novelty of this study.

Calcium, magnesium, phosphorus and carbon analyses of the samples are shown in Table 3. The sixth column (i.e., Ca/P molar ratio) of Table 3 is calculated from the data of 4th and 5th columns. All samples had a Ca/P molar ratio between 1.28 and 1.32 while containing

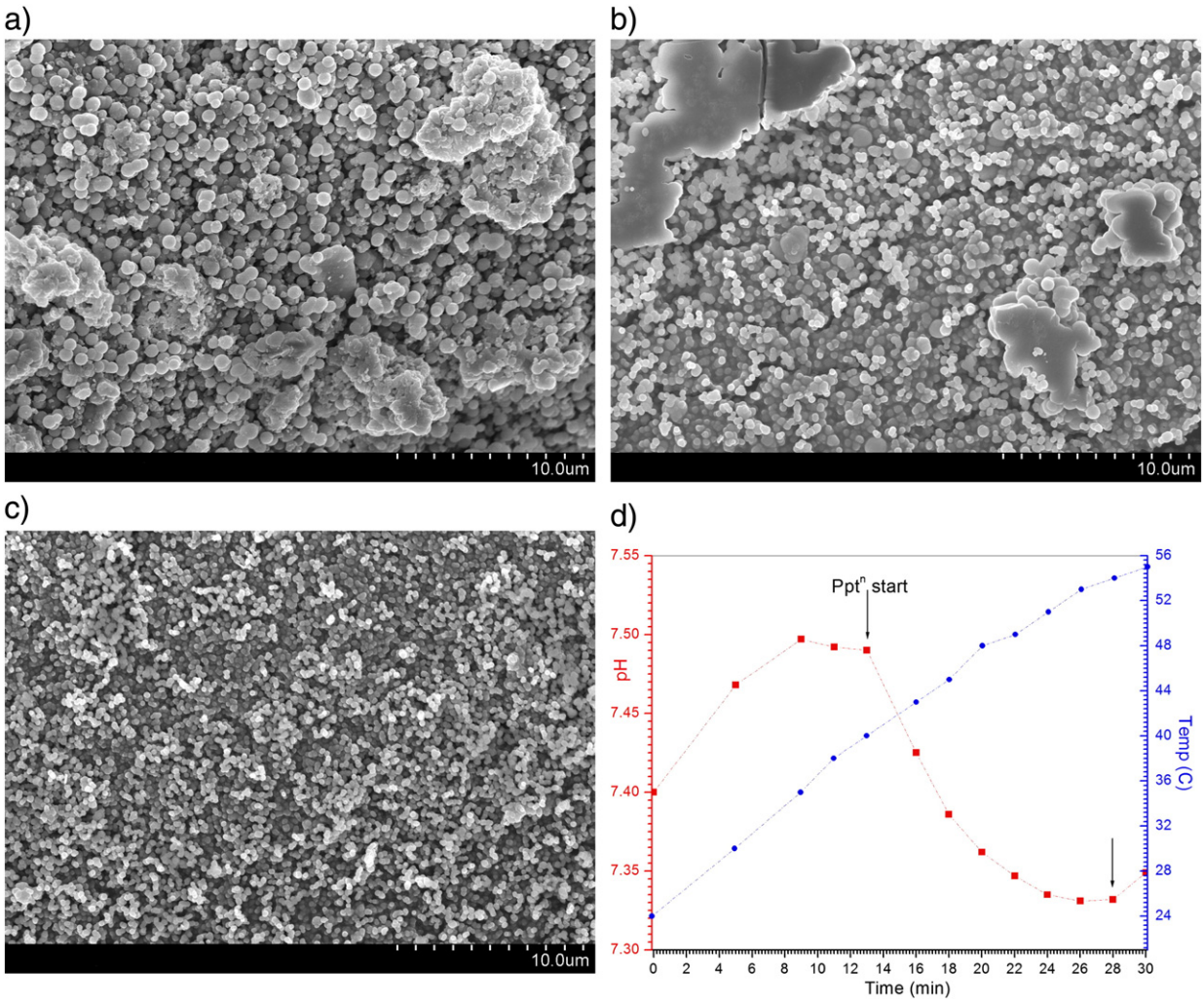

Fig. 2. (a) SEM image of ACP samples synthesized in *Lac*–SBF at 65 °C, (b) SEM image of ACP samples synthesized in *Lac*–SBF at 70 °C, (c) SEM image of ACP samples synthesized in *Lac*–SBF at 55 °C, and (d) pH versus time–temperature chart of a typical sphere synthesis at 55 °C in *Lac*–SBF.

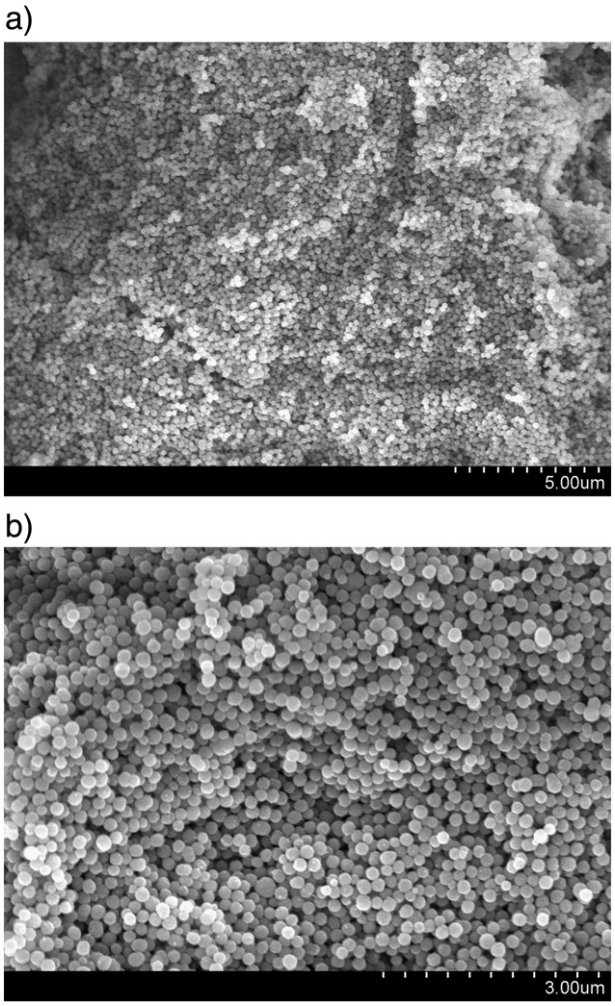

Fig. 3. SEM photomicrographs of ACP spheres obtained at 55 °C (850 rpm) in a *Lac*–SBF solution containing 67 mg/L gelatin; (a) low, and (b) high magnification.

magnesium and carbon. The ACP particles synthesized were Mg-doped and carbonated. That 1600 ppm Mg present in the ACP samples would be quite difficult to detect by using routine energy-dispersive X-ray spectroscopy (EDXS) analyses. The carbon percentages of Table 3 correspond to about 4 wt.% CO_3 in the ACP samples. For the sake of analogy, the mineral portion of adult human bones contains 5.8 wt.% CO_3 [18].

4. Discussion

The *Lac*–SBF solution of this study flawlessly matched the inorganic ion concentrations of the human extracellular fluid (ECF) and/or blood plasma, and upon simultaneous heating and stirring at 55 °C for only 15 min, these solutions produced monodisperse spheres of X-ray-amorphous CaP (ACP). The use of SBF solutions at temperatures above 37 °C to synthesize monosize ACP spheres was not reported before.

Although Posner et al. [19] were among the first to synthesize ACP in water, they did not perform their ACP syntheses in solutions simultaneously containing Na^+, Mg^{2+}, K^+, Cl^- and HCO_3^- at concentration levels similar to those of ECF (extracellular fluid). ACP powders synthesized in water were comprised of non-spherical particles of irregular shapes, as previously shown, for instance, in Fig. 11 of the report of Eanes and Meyer [20].

Attempts to synthesize CaP in solutions similar to the physiological solutions (i.e., ECF and/or blood plasma) have always been quite limited and such syntheses were mostly performed at 37 °C. Any literature reports on the synthesis of CaP particles in physiological solutions heated at 55 to 70 °C have been difficult to find.

Bachra et al. [21,22] reported in 1962 and 1963 the synthesis of calcium phosphates at 37 °C and pH 7.3 in solutions which partially mimic the inorganic ion concentrations of ECF. The solutions of Bachra et al. [22] contained 145 mM Na^+, 133 mM Cl^-, 5 mM K^+, 3.75 mM Ca^{2+}, 1.67 mM P, 22 mM HCO_3^- and 3 to 10 mM Mg^{2+}. Calcium ion, HPO_4^{2-} and Cl^- concentrations of such solutions were far away from those of ECF. Bachra et al. [22] aged the solutions statically (i.e., no stirring) at 37 °C from 5 to 72 h and reported the formation of ACP precipitates when they increased the Mg^{2+} concentration from 1 mM to 3 mM and above, while studying the HCO_3^- concentrations of 22 and 110 mM at such Mg levels. Termine and Eanes [23], on the other hand, used the Earle's balanced salt solution (EBSS) as the medium for synthesizing calcium phosphates at pH 7.4 and 37 °C. EBSS solution is not able to match the ion concentrations of ECF by having 143.6 mM Na^+, 5.37 mM K^+, 0.83 mM Mg^{2+}, 1.8 mM Ca^{2+}, 1.04 mM HPO_4^{2-}, 125.3 mM Cl^-, 0.83 mM SO_4^{2-} and 26.2 mM HCO_3^-. Termine and Eanes [23] buffered the EBSS using 25 mM Hepes at pH 7.4 (at 37 °C), and then added the salts of $CaCl_2$ and Na_2HPO_4 at the level of 2.1 mM^2, in terms of CaxP molar product, to initiate the synthesis. Termine and Eanes [23] thus synthesized ACP spherules with diameters in the vicinity of 100 nm within the first 24 h of aging their solution at 37 °C.

Tas et al. [24–26] and Landi et al. [27] independently synthesized ACP nanoparticles of irregular shapes by heating 50 mM Tris/HCl-buffered, 125 mM Cl^-- and 27 mM HCO_3^--containing SBF solutions again at 37 °C. Fig. 4 shows the characteristic ACP precipitates obtained in a 27 mM HCO_3-containing Tris/HCl-buffered SBF solution heated at 37 °C for 48 h (stirred at 50 rpm) [25]. It takes quite a long time to form ACP precipitates in the SBF solution heated at 37 °C. The preparation conditions of this solution and its heating at 37 °C, together with the XRD and FTIR characterization results, were published previously [25].

The novelty of the current study is its ability to produce monodisperse submicron ACP spheres at 55 °C. The *Lac*–SBF solution had sufficient concentrations of ACP-stabilizing aqueous ions (such as Mg^{2+} and HCO_3^- [28–30]). The uniform ACP spheres of this study are formed as a result of the simultaneous contribution of several factors;

(i) increasing the solution temperature to such a value (e.g., 55 °C) that the solution would no longer be stable with respect to the rapid coalescence and aggregation of Posner's clusters (initially being in the colloidal size range, with diameters close to 1 nm [31–33]),
(ii) constant stirring of the solution at a high rate of 850 rpm,
(iii) forming the spheres in the presence of biological macromolecules

Table 3
Results of quantitative chemical (ICP-AES) analyses (in wt.%).

Sample	Mg	C	Ca	P	Ca/P molar ratio
55 °C	0.161 ± 0.02	0.832 ± 0.06	28.02 ± 0.03	16.66 ± 0.01	1.30
65 °C	0.165 ± 0.03	0.818 ± 0.07	27.94 ± 0.02	16.87 ± 0.03	1.28
70 °C	0.159 ± 0.03	0.864 ± 0.06	28.11 ± 0.03	16.84 ± 0.02	1.29
55 °C w/gelatin	0.160 ± 0.04	0.911 ± 0.06	27.98 ± 0.03	16.38 ± 0.02	1.32

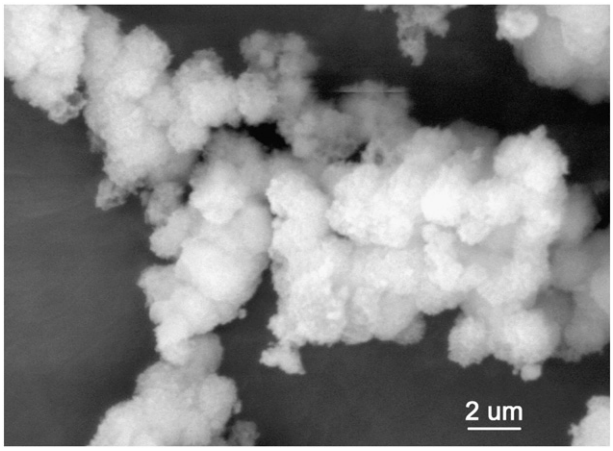

Fig. 4. SEM photomicrograph of characteristic ACP agglomerates synthesized in an SBF solution at 37 °C (50 rpm).

provided by bovine gelatin, which is a balanced concoction of amino acids [34,35].

The first feature of the above process is the heating of the *Lac*–SBF solution to above 37 °C, which resulted in the observation of a bluish tint in solution at 40 °C. The period of smooth pH decrease (via Eq. (1b)) from 40 to 55 °C (Fig. 2d) refers to the hydrothermal decomposition of solution. The solution became milky white and turbid at above 50 °C. This milky white state means that upon heating the *Lac*–SBF solution to 55 °C, a significant amount of the aqueous ions of Ca^{2+}, Mg^{2+}, HCO_3^- and HPO_4^{2-} participated in forming the X-ray-amorphous particles. It is a well-established fact that SBF solutions never display this much turbidity when heated only to 36.5–37 °C [4,8].

The second aspect of the synthesis process is the agitation (i.e., stirring) of solution during the entire course of reaction. The agitation continues since the onset of colloidal particle formation (bluish tint) until the spheroidization of ACP precipitates. Stirring a solution at 850 rpm, versus stirring at 50 rpm (Fig. 4), helps to reduce the likelihood of forming large agglomerates (>10 μm) of precipitates. The observation of ice boulders (or balls) [36] on the shores of, for instance, Lake Michigan is how nature forms monodisperse balls of ice each weighing 25 kg. The water temperature on the Lake Michigan drops below freezing, so when a small piece of ice is forming in the water waves move (and rotate) it back and forth to add more ice crystals. These boulders swimming and rotating in water gets bigger and bigger, and eventually they are pushed to the shore by the wind. A few hundred of Posner's clusters, initially present in any SBF solution [31–33], coalesce to form a single ACP particle having a diameter of 245 to 300 nm when the solution was heated at 55 °C under constant stirring.

Brecevic et al. [34] studied the presence of gelatin (typically at 400 to 1500 mg/L levels) in decreasing the ACP particle size and their state of agglomeration. They performed their study in solutions (at pH 7.4, regulated with NaOH additions, and at room temperature) simultaneously containing dissolved calcium chloride and sodium phosphate salts. Brecevic et al. [34] categorized the ACP-gelatin interaction by one or more of the following processes; adsorption of gelatin macromolecules on the ACP particle surface, nucleation of ACP on the gelatin macromolecules or the occlusion of gelatin inside the ACP particles. The last of these options seemed unlikely, if the gelatin macromolecules were occluded in the ACP particles, we should have observed the amide bands in our FTIR data. Brecevic et al. [34] obtained experimental support, through zeta potential measurements, to the scheme of gelatin macromolecule adsorption on the ACP particles. The adsorption of gelatin macromolecules on monodisperse $CaCO_3$ micropills or microtablets was previously shown to be possible during syntheses performed in $CaCl_2$-gelatin-urea solutions at 70 °C [37,38]. Nevertheless, in case of a possible deposition of other CaP phases, such as apatitic CaP or octacalcium phosphate (OCP, $Ca_8(HPO_4)_2(PO_4)_4{\cdot}5H_2O$), on the already present ACP particles, the occlusion of gelatin macromolecules in between the ACP particle surface and the crystals of the deposited phase may be a viable process to consider.

The study of Brecevic et al. [34] showed that it is possible to decrease the ACP particle diameter down to the range of 5–10 nm, by increasing the dissolved gelatin concentration in solution to around 1500 mg/L. The gelatin concentration of the current study was only 67 mg/L, which resulted in 245 nm particles. The concentrations of specific amino acids present in bovine gelatin, on the other hand, were tabulated by Arnesen and Gildberg [35]. The process yield for ACP synthesis described in this study may be increased by using, for instance, a 10× SBF solution recipe [39,40].

Such biocompatible and non-cytotoxic ACP spheres may find use in oncological and pharmaceutical research as drug delivery agents.

5. Conclusions

A recently developed SBF solution (*Lac*–SBF) faultlessly matching the inorganic ion concentrations of the blood plasma, which is buffered with Na-L-lactate/lactic acid, was used for the first time as the solution to in situ synthesize submicron X-ray-amorphous calcium phosphate (ACP) spheres. The *Lac*–SBF solution containing a small amount of gelatin (67 mg/L) autogenously produced monodisperse ACP particles with diameters less than 250 nm upon heating and stirring it at 55 °C for 15 min.

6. Notes

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

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