Journal of Non-Crystalline Solids 400 (2014) 27–32

Contents lists available at ScienceDirect

Journal of Non-Crystalline Solids

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

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

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ARTICLE INFO

Article history:
Received 6 April 2014
Received in revised form 25 April 2014
Available online 20 May 2014

Keywords:
Amorphous;
Calcium phosphate;
Submicron;
Spheres;
Synthesis

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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http://dx.doi.org/10.1016/j.jnoncrysol.2014.04.031
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

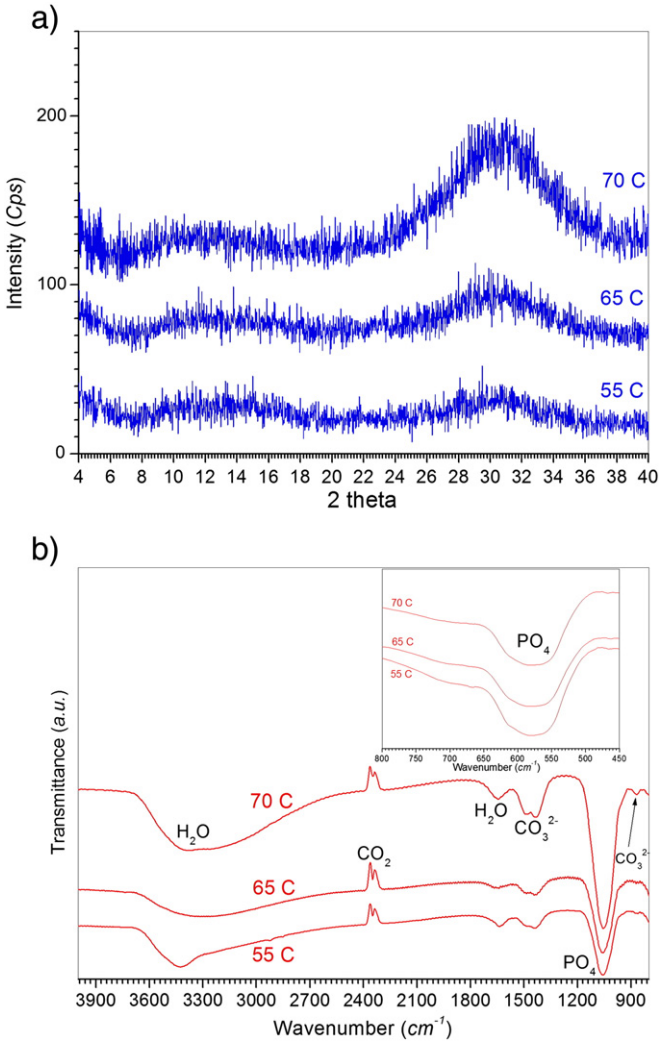

Fig. 1. (a) XRD and (b) FTIR spectra of ACP (amorphous calcium phosphate) samples synthesized at different temperatures in *Lac*–SBF.