Formation of hydroxyapatite precursors at 37 °C in urea- and enzyme urease-containing body fluids

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Calcium hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$) bioceramics, owing to their similarity with the human bone and dentin minerals, attract significant interest for orthopaedic and dental applications. Biological apatites, on the other hand, were observed [1] to be carbonate-substituted and calcium-deficient. HA powders for bioceramic applications have usually been chemically synthesized [2–9] via aqueous solutions. It is known [6] that HA is the least soluble and the most stable compound of calcium phosphate phases in aqueous solutions at pH values higher than 4.2. HA powders synthesized in highly alkaline (pH > 0) media [2–9] were typically recognized by their relatively high thermal stability and phase purity even after high temperature (1100–1300 °C) sintering. However, formation of HA powders in neutral and/or slightly acidic aqueous media is known to be a more complicated and difficult task [7, 10].

Simulated body fluids (SBF), with ion concentrations resembling those of human blood plasma, were first used by Kokubo *et al*. [11, 12], 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 apatite, which was similar to bone apatite [12].

Biomimetic HA powders were previously synthesized by Tas [13] from calcium nitrate tetrahydrate and di-ammonium hydrogen phosphate salts dissolved in synthetic body fluid solutions (instead of distilled water), under the physiological conditions of 37 °C and pH 7.4. The maintenance of pH value at or slightly above 7.4 during precipitation required [13] a controlled but continuous addition of a base, such as diluted solutions of NH_4OH. The addition of urea (H_2NCONH_2) into the body fluids just before the HA precipitation experiments was found [14] to cause a significant reduction in the amount of the base which needed to be continuously added to keep the pH at 7.4. The procedure of addition of enzyme urease (which is known [15] to accelerate the decomposition rate [16] of urea, especially at low temperatures) into the urea-containing body fluids, and the use of these in the synthesis of biomimetic HA precursors at 37 °C and pH value of 7.4 are hereby presented.

Body fluids were prepared by dissolving reagent-grade chemicals of NaCl, $NaHCO_3$, KCl, $Na_2HPO_4 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, Na_2SO_4, $(CH_2OH)_3CNH_2$, and $CaCl_2 \cdot 2H_2O$ in de-ionized water (see Table I), exactly as described previously by Tas *et al*. [13, 14].

0.16 mol of urea was first dissolved in 205 mL of body fluid at room temperature, followed by addition of a 0.4 mL aliquot of a methyl cellulose (i.e., as dispersant) stock solution (1 gram/L). The temperature of the reaction beaker was raised to 37 °C in a microprocessor-controlled water bath. Enzyme urease (Merck, 5 U/mg), over the range of 102.5 mg (2.5 U/mL) to 410 mg (10 U/mL), was then added into the above solution. Formation of hydroxyapatite precursors in this solution was finally achieved by adding, in powder form, 0.012 mol of $(NH_4)_2HPO_4$ and 0.0201 mol of $Ca(NO_3)_2 \cdot 4H_2O$, at once. The opaque solution was mixed and aged at the constant temperature of 37 °C for about 3 h. Precipitates were recovered from their mother liquors by filtration and washed five times with de-ionized water, whose pH was pre-adjusted to 7.4. Powders were then calcined in air over the temperature range of 300–1100 °C for 6 h, following their drying at 90 °C. Samples were characterized by XRD, SEM, ICP-AES, and FTIR.

The use of urea and enzyme urease-containing body fluids in the biomimetic synthesis of HA precursor powders at 37 °C has been attempted for the first time in this study. In case of successful HA synthesis by using the starting chemicals of $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ dissolved in water, it is known [2–9] that the solution pH values must be in the alkaline region. On the other hand, in the case of using body fluids as the synthesis medium [13, 14], the pH value needed for HA formation may be reduced considerably. Following the addition of enzyme urease into the urea-containing

TABLE I Ion concentrations of body fluids and human plasma

Ion	Kokubo[11] (mM)	This study (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

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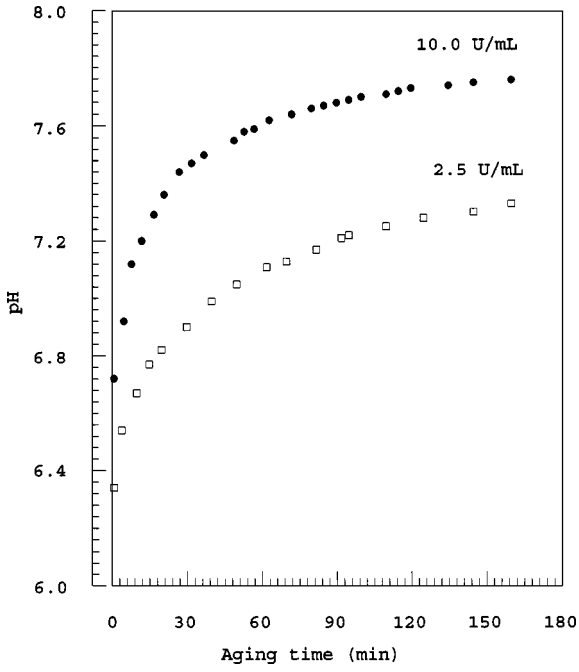

Figure 1 pH control provided by urease (at two different concentrations) in urea-containing body fluids during precursor formation at 37 °C.

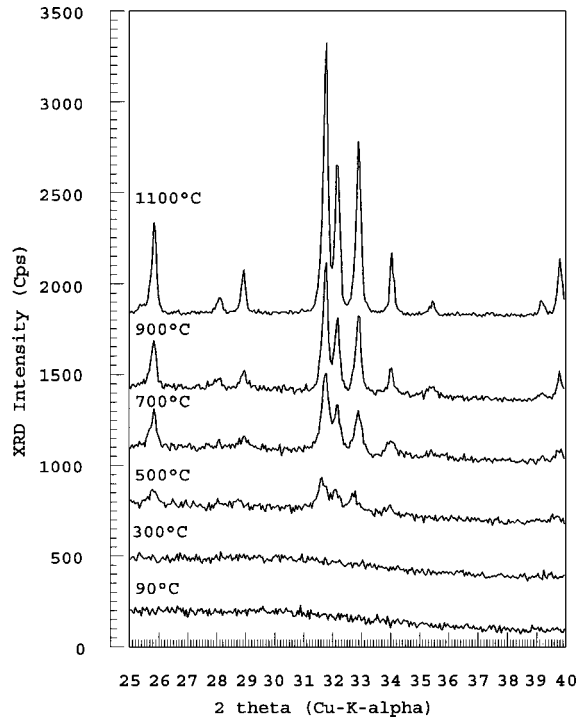

Figure 2 XRD spectra showing the crystallization of precursors.

body fluids (i.e., time = 0), the pH values have changed as shown in Fig. 1, as a result of accelerated decomposition of urea [16]. When the enzyme concentration was kept at the initial, nominal level of 2.5 units/mL, the synthesized powders consisted of two phases, i.e., a mixture of HA (80%) and $Ca_3(PO_4)_2$ (20%). However, when the enzyme concentration was increased to 10 units/mL, the solution pH was observed to rise to the vicinity of 7.4, in about the first 15–20 min of aging at 37 °C. The powders obtained at this concentration of urease were found to be single phase HA after calcination at 500–1100 °C. The biomimetic pH control in urea-containing body fluids is thus shown to be easily achievable, at 37 °C, by the addition of the enzyme urease in appropriate amounts (8–10 units/mL). The necessity for strong base additions (e.g., NH_4OH) to the precipitation solutions are therefore totally eliminated.

Calcium phosphate precipitates formed at 37 °C and pH of 7.4 in urea- and enzyme urease-containing body fluids were found to be amorphous, following overnight drying at 90 °C. Crystallization of these precursors started at above 300 °C, and after calcination at 1100 °C for 6 h, they completely transformed into single-phase HA. Fig. 2 shows the crystallization behavior of these powders. HA powders calcined at 1100 °C were found to have the lattice parameters of $a = 9.420$ and $c = 6.885$ Å, with a unit cell volume of 529.04 Å^3. These powders also contained small amounts of other inorganic ions (just like natural bones), provided by the use of body fluids. ICP-AES analyses performed on 1100 °C-calcined samples indicated the presence of about 1500 ppm Mg and 160 ppm Na. These ions are shown, by Rietveld analysis [14], to incorporate themselves into the crystal structure of calcium hydroxyapatite. Powder bodies calcined at 1100 °C consisted of particles in the size range of 20 to 50 nm as shown in the SEM micrograph of Fig. 3. The FTIR spectrum of the powders of Fig. 3 is given in Fig. 4. The CO_3^{2-}

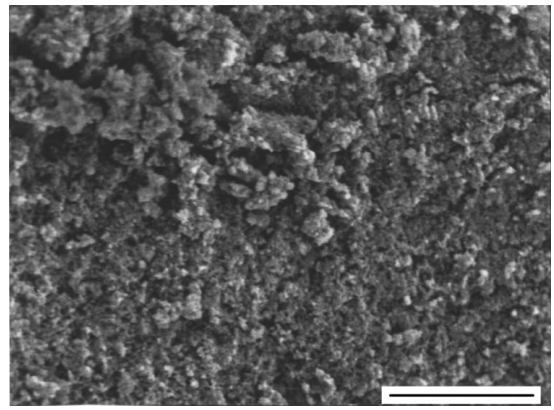

Figure 3 SEM micrograph of HA powders calcined at 1100 °C (Bar = 1 μm).

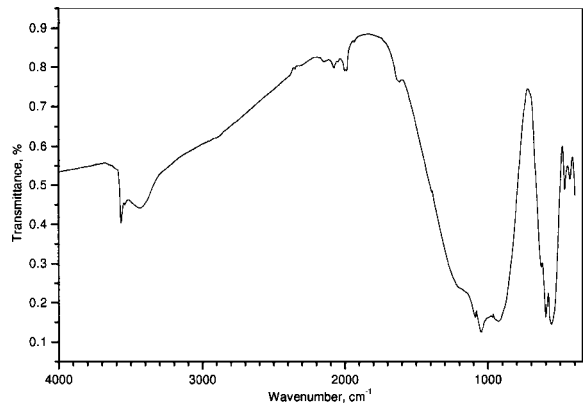

Figure 4 FTIR spectrum of HA powders calcined at 1100 °C.

ion peaks, at 2368-2361 (combination of $\nu_2 + \nu_3$), 1467-1412 and 878 cm^{-1}, are observed. The OH^- stretching vibration at 3571 cm^{-1} and the OH^- bending vibration at 635 cm^{-1} were seen. The PO_4 bands were detected at 470 (ν_2), 570 and 603 (ν_4), 962 (ν_1), 1045 and 1096 (ν_3) cm^{-1}.

Enzyme urease when added in proper amounts into the urea-containing body fluids (which are used for HA precursor synthesis) is shown to supply a plausible and automatic pH control to be achieved at 37 °C, totally eliminating the need of external base additions.

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