

BIOMIMETIC PREPARATION OF HA PRECURSORS AT 37°C IN UREA- AND ENZYME UREASE-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 and sub-micron bioceramic powder. Carbonated HA precursors were synthesized from calcium nitrate tetrahydrate and diammonium hydrogen phosphate salts dissolved in "synthetic body fluid" (SBF) solutions, containing urea (H_2NCONH_2) and enzyme urease, under the biomimetic conditions of 37°C and pH 7.4, by using a novel chemical precipitation technique.

INTRODUCTION

Calcium hydroxyapatite (HA: $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), the main inorganic component of human 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 observed [1] 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 for bioceramic applications have generally been synthesized by using aqueous solutions. It is known [4] 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 study [12] will focus on the determination of the experimental parameters of HA precursor synthesis at the physiological pH (7.4) and temperature (37°C), in SBF solutions rich in urea and enzyme urease.

Merck (Darmstadt, Germany)-grade NaCl (99.5%), NaHCO₃ (99.5%), KCl (99.0%), Na₂HPO₄·2H₂O (99.5%), MgCl₂·6H₂O (98.0%), Na₂SO₄ (CH₂(OH)₂CNH₂ (99.2%), CaCl₂·2H₂O (99.0%) and HCl (37 vol%, Carlo-Erba, Italy) were used in the preparation of the synthetic body fluids. SBF solutions were prepared [12] by dissolving appropriate quantities of the above chemicals in de-ionized water. A total of 40 mL of 1M 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 appropriately titrated with 1M HCl to the pH value 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.

0.4 M Ca(NO₃)₂·4H₂O (Riedel de-Haen, 99%, Germany) and 0.16 M (NH₄)₂HPO₄ (Merck, 99%, Germany) stock solutions were used in the precipitation experiments as the calcium and phosphate ion sources. 8 to 9.5 M urea solutions were prepared (by dissolving the urea powder in SBF) and then aged at 85°C overnight in a sealed glass jar, to ensure the decomposition of urea. The pH values of 9.5 M, "aged urea" solutions were measured as 9.3 at 37°C, prior to their use in powder synthesis. The enzyme urease (Merck, Lot No: 108489, 5 units/mg, Germany) was added, within the concentration range of 2.5 to 10 units/mL, into the precipitation solutions to establish and accelerate [13] the complete decomposition of urea at 37°C.

The pH values of the solutions were maintained [12] at the physiological level of 7.4 during the entire chemical precipitation experiments. The synthesized powders were calcined in an air atmosphere over the temperature range of 1100°-1600°C for 6 to 17 hours, following their drying at 80°C.

The phase purity and the levels of crystallinity of the calcined HA powders were studied by powder X-ray diffraction (Rigaku, Model: D-Max/B, Tokyo, Japan) at the typical step size of 0.05° 2θ and a count time of 1 s. A monochromated 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. Further details of XRD procedures were described elsewhere [14].

Fourier-transformed infra-red spectroscopy (FT-IR) (Nicolet, Model: DX-510, WI, USA) was used in the wave number range of 4000-400 cm⁻¹. Experimental spectra of solid samples were obtained by preparing KBr pellets with a 3:97 (wt%) sample-to-KBr proportions. Inductively-coupled plasma atomic emission spectroscopy (ICP-AES) (Perkin Elmer, Model Plasma-1000, 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 the synthesis experiments.

RESULTS AND DISCUSSION

The nominal 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 this study, as compared to the previous workers, in terms of "SBF ion concentrations" can be stated as follows: (a) the nominal, initial HCO₃⁻ ion concentration of SBF was increased from 4.2 mM to 27.0 mM which, thus, acquired exactly the same value with that of human plasma, (b) Cl⁻ ion concentration was decreased from 147.8 mM to 125.0 mM in the preparation recipe, whereas the Cl⁻ ion concentration of human plasma is 103.0 mM. This has solely been achieved [12] by changing one of the reagents of SBF preparation recipe from K-phosphate [2, 3] to Na-phosphate.

The as-filtered, dried and nano-sized (as shown in Figure 1) calcium phosphate precipitates formed at 37°C and pH of 7.4 via urea-containing SBF solutions were found [12] to be amorphous. Upon calcination of these amorphous precursors, in a stagnant air atmosphere, at 1100°C for 6 hours, 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 tricalcium phosphate (Ca₃(PO₄)₂), calcium pyrophosphate (Ca₂P₂O₇), tetra calcium phosphate (Ca₄P₂O₇), and/or into oxyapatite (Ca₁₀(PO₄)₆O) when heated above 1200°C in air. The HA precipitates [12] formed in SBF solutions had an excellent thermal stability even when heated at 1600°C for 6 hours in an air atmosphere. Figure 2 shows the XRD traces of SBF-synthesized HA precipitates heated at different temperatures, over the range of 1300° to 1600°C, for 6 hours. The XRD spectra of all samples closely matched with the ICDD PDF No: 9-432 for calcium hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂). HA powders of high thermal stability are expected to be promising materials especially for the high-temperature requiring implant manufacturing processes, such as plasma-spraying of ceramic powders on to metals.

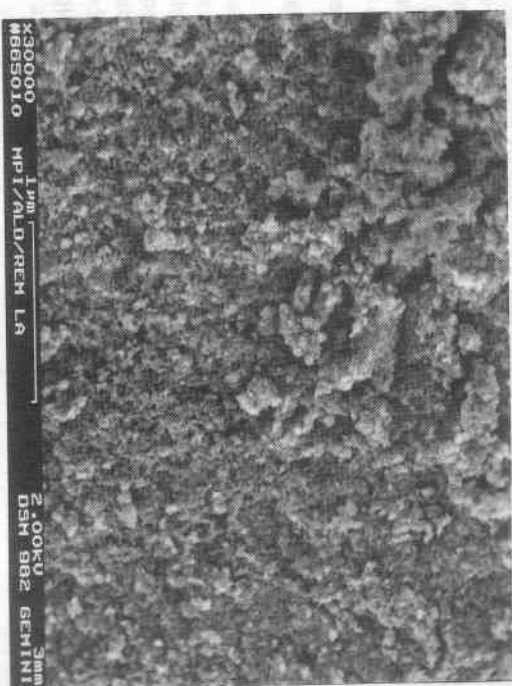


Fig. 1 SEM micrograph of HA powders formed in Urea/Urease-cfg SBF at 37°C

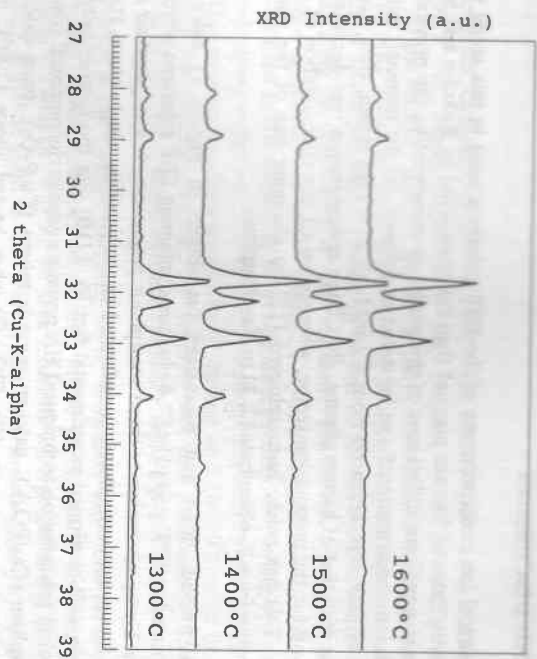


Fig. 2 High-temperature behavior of Urea/Urease-SBF synthesized HA powders

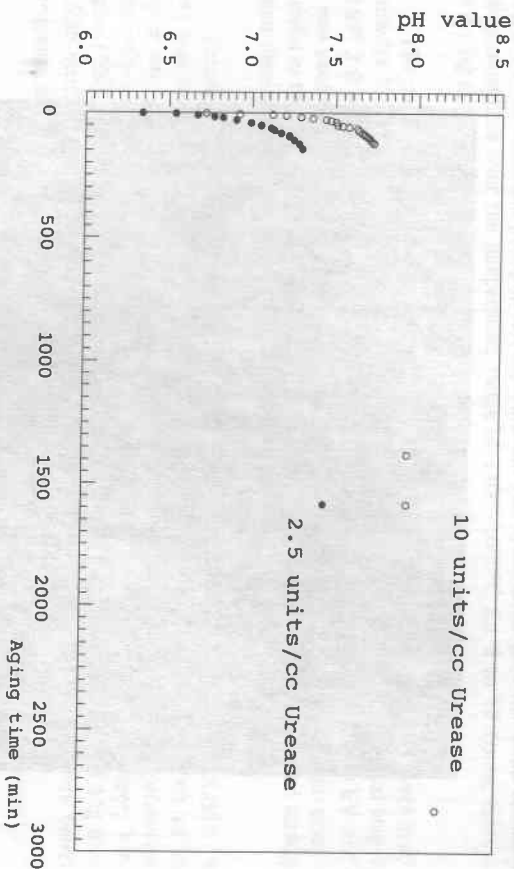


Fig. 3 pH control established by enzyme urease in SBF solutions at 37°C

Biomimetic HA powders of the present study [15] also contained trace amounts of other inorganic ions, provided by the use of SBF solutions. ICP-AES analysis performed on 1150°C-calcined samples indicated that these HA powders had 1500 ppm Mg and 160 ppm Na. The powders did not contain any nitrogen, the organic material volatilized completely during calcination at 500°C. Na⁺ and Mg²⁺ ions have been originated from the use of SBF solutions during precipitation. The lattice parameters of these SBF-grown powders were measured as a = 9.417 and c = 6.876 Å. The hexagonal unit cell (of the space group P6₃/m) volume was found to be equal to 527.95 Å³.

The FT-IR spectra of SBF-synthesized HA powders (after 6 hours of calcination at 500°C in air) clearly showed the CO₃²⁻ bands at 2368-2361 (combination of ν₂ + ν₃), 1467-1412 and 878 cm⁻¹. As the calcination temperature increased (>850°C), these bands disappeared. The OH stretching vibration was observed at 3571 cm⁻¹ and the OH bending vibration recorded at 635 cm⁻¹. The PO₄ bands were detected at 470 (ν₂), 570 and 603 (ν₄), 962 (ν₁), 1045 and 1096 (ν₃) cm⁻¹.

In the HA synthesis practices which used the starting chemicals of Ca(NO₃)₂·4H₂O and (NH₄)₂HPO₄, it was known that if the synthesis medium was pure water, then the solution pH values must be in the alkaline region [6-11]. On the other hand, in the case of using SBF, the pH value needed for HA synthesis decreases [12, 15] considerably. In combination with the initial presence of spontaneously formed seed crystals 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 precursor phase that would otherwise, normally, not nucleate at such a low pH value of 7.4. Following the addition of the enzyme urease into the precipitation solutions, the pH values have changed as shown in Figure 3. (Without the addition of enzyme urease into the same solutions, the plots would look like horizontal, straight lines.) When the enzyme concentration was kept at the initial, nominal level of 2.5 units/mL, the synthesized powders were consisted of two phases, i.e., a mixture of HA (20%) and Ca₃(PO₄)₂ (20%). However, when the enzyme concentration was increased to 10 units/mL, the solution pH value was observed to rapidly rise to ~7.4, in about the first half hour of aging at 37°C. The powders obtained at this concentration of urease were found to be single phase HA after calcination at 1000°C. The biomimetic pH control in urea-containing SBF's have thus been shown to be achievable, at 37°C, by the addition of the enzyme urease in appropriate amounts (2-3.5 units/mL).

CONCLUSIONS

HA powders have been synthesized by a novel chemical precipitation technique via urea/urease-containing synthetic body fluid solutions, at the physiological conditions of pH 7.4 and 37°C, with dissolved calcium nitrate tetrahydrate and di-ammonium hydrogen phosphate salts in appropriate amounts. Enzyme urease, when added in proper amounts into the urea-containing synthetic body fluids used for HA synthesis, was shown to supply a plausible pH control required for the achievement of biomimetic conditions. The produced HA powders were shown to be carbonated and to have unprecedented phase stability even when heated at 1600°C, for 6 hours, in a stagnant air atmosphere.

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ABSTRACT

New bone formation around implants is one of preferable conditions for the successful implantation. In this study, formable carbonate apatite ceramics were prepared and implanted in cavities (ϕ 400 μ m, depth 400 μ m) on the surface of pure titanium implant (ASTMB348-GR2) to enhance new bone formation around the titanium implant.

INTRODUCTION

Implants for loaded position such as artificial tooth roots and artificial hipbone are made of titanium and some of its alloys to achieve sufficient mechanical strength and low-cytotoxicity. One of keys for the successful titanium implantation is fixation of the implants to bone. Enhancement of new bone formation around the implants has been providing favorable prosthodontic solution [1]. One of these attempts is hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HAP) coating on the surface of the implants. HAP is a main inorganic component of bone and teeth and induces new bone formation [2-6]. However, HAP coating is a kind of ceramics thin layer with brittleness, meaning high susceptibility to a tiny defect, although HAP coating contributes to initial fixation of the implants [7-9]. Therefore, establishment of stable apatite fixation on the implants has been a deep interest. This study presented new method to fix apatite on the surface of the titanium. Formable carbonate apatite (CHAP) ceramics were prepared and implanted on the surface of pure titanium at 1023 K.

EXPERIMENT

Preparation of carbonate apatite ceramics

Carbonate apatite was prepared by mixing 8 L of 2 mol/L calcium nitrate solution and 2 L of 1.2 mol/L disodium hydrogen phosphate solution containing 6 mol disodium carbonate for 3 days at 373 K and pH 9.0 - 0.1 [10]. The precipitated carbonate apatite was centrifuge washed 10 times with ultra pure water, and was isolated by decantation, followed by drying at 383 K using drying oven. The isolated carbonate apatite was sintered at 1023 K, then crushed into 500 μ m mesh polygonal pieces. Carbonate apatite ceramics prepared in the manner were porous and showed plastic formability at near 1023 K. Carbonate content of the carbonate apatite ceramics was 6 wt %.

Preparation of pure titanium substrates

Pure titanium (ASTMB348-GR2) was cut into a dimension of $10 \times 10 \times 5$ mm for the substrate, then polished with No. 900 diamond polishing sheets. Twenty cavities for the carbonate apatite implantation were grooved on each of two parallel 10×5 mm face of the