CHARACTERIZATION OF NANOHYDROXYAPATITE PREPARED BY PRECIPITATION FROM CALCIUM $\alpha$-D-HEPTAGLUCONATE PRECURSOR SOLUTIONS

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Abstract
Calcium deficient nanohydroxyapatite (nanoHap) powders were synthesized by co-precipitation of calcium $\alpha$-D-heptagluconate hydrate and diammonium hydrogen phosphate solutions. TEM and HRTEM images of powders after the drying of products showed that the reactant concentration affected the sizes and morphology of particles. Small changes were found in phase composition of nanoHap powders synthesized from variously concentrated reactant solutions. The biphasic mixtures of hydroxyapatite and $\beta$-tricalcium phosphate ($\beta$-TCP) were formed after annealing at 1000°C.

Keywords: hydroxyapatite, morphology, crystallinite size, nanoparticles, HRTEM

INTRODUCTION
Hydroxyapatite (Hap) as the main inorganic component of bones and teeth has been synthesized by various methods and at different reaction conditions [1-4]. The low temperature precipitation represents the most common method for Hap preparation owing to its easy in experiment operation, variability in final Hap crystallinity, size and morphology [5]. Hap in bones is present in the form of nanometer-sized needle-like crystals of approximately 5-20 nm width and 60 nm in length. The biological advantages of employing nano Hap lies in its specific affinity towards many adhesive proteins and its direct involvement in bone cell differentiation and the mineralization process. In general, during the precipitation process of calcium phosphates in aqueous solution, an amorphous phase – amorphous calcium phosphate (ACP) is readily created and transformed directly into more stable nanocrystalline Hap [6]. The conversion mechanism of ACP-Hap has been studied by many authors and in various environments e.g. in the state of suspensions and gel-like states, on just-precipitated wet samples and on lyophilised and heated powders [7,8]. In practice, the hydroxyapatite can be prepared by neutralization and precipitation methods, which could be modified by the addition of other substances like, for example, EDTA [9], citric acid [10], polyacrylic acid [11] etc. Poinern et al. [12] prepared nano Hap by wet chemical precipitation using Ca(NO$_3$)$_2$·4H$_2$O and KH$_2$PO$_4$ as the Ca and P sources respectively. They obtained spherical particles of the nanometric size approximately 30 nm. Similarly, the same precursors have been used by Yubao et al. [13] and a biphasic hydroxyapatite and $\beta$-tricalcium phosphate (Hap+$\beta$-TCP) mixture with rod-like particle morphology. A nanosized rod like Hap crystals were produced by Huang et al. [14] from calcium hydroxide and orthophosphoric acid precursors. Their results indicated that the nano Hap crystals are likely to produce favourable biological responses in vivo. Rajabi-Zamani et al. [15] examined non-aqueous preparation of hydroxyapatite from non-alkoxide

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precursors in ethanol, where the carbonated hydroxyapatite was formed. Tung et al. [16] showed that the amorphous calcium phosphates are created in ethanol-water solutions and the ACP’s rapidly transform to poorly crystallized hydroxyapatite at pH 7.4.

In this work the effect of calcium α-D-heptagluconate solution concentration on the particle size, morphology and phase composition of precipitated nanoHap was investigated. The utilization of calcium α-D-heptagluconate as an organic Ca$^{2+}$ source for the hydroxyapatite preparation is advantageous because of its non-toxic and body-friendly behaviour. The synthesized nanoHap can be applied in native powder form as the inorganic filler in composites and traces of organic ligands, which could be adsorbed on a nanoHap surface, do not cause any health risk after implantation.

EXPERIMENTAL

NanoHap powders were synthesized by the co-precipitation of calcium α-D-heptagluconate hydrate (≥98%, Fluka) and diammonium hydrogen phosphate ((NH$_4$)$_2$HPO$_4$ analytical grade, Merck) solutions at three different concentrations - 0.200 mol dm$^-3$ (high Hap), 0.104 mol dm$^-3$ (medium Hap) and 0.052 mol dm$^-3$ (low Hap) for both precursors. These solution concentrations represent supersaturated, saturated and undersaturated solutions of calcium α-D-heptagluconate hydrate. The calcium α-D-heptagluconate hydrate solutions were slowly dropped (3 ml/min) to the diammonium hydrogen phosphate solutions at room temperature. The mixtures were stirred at 400 rpm using the magnetic stirrer (HEIDOLPH MR Hei-Tec). The mole ratio of Ca/P in the final mixtures was 1.67 and the pH was kept at 10.0 by an addition of NH$_3$ (aq). The precipitates were aged for 7 days in distilled water, washed with de-ionized water and ethanol; filtered over the membrane filter (Millipore, 0.2 μm pore size) and dried at 105°C for 4 h. Then the powders were crushed, sieved (Mesh 250) and annealed at 1000°C for 30 min in air.

The phase composition of samples was determined by X-ray diffraction analysis (XRD, Philips X’PertPro, Cu Ka radiation) and infrared spectroscopy (FTIR Shimadzu IRAffinity1, 300 mg KBr+1 mg sample). The morphology and particle size of the powder samples were observed by the transmission electron microscopy (JEOL JEM 2100 F). The final Ca/P ratio in precipitates was determined by the chemical analysis after their dissolution in diluted HNO$_3$ (analytical grade, 1+3), where calcium was determined by the complexometry (EDTA) and phosphorus as the P–Mo–V complex by the colorimetry. The pH values of solutions were adjusted using a pH-meter (WTW, Inolab 720) with the combined electrode SenTix 41.

RESULTS AND DISCUSSION

The TEM and HRTEM micrographs of dried nanoHap particles prepared at various reactant concentrations are shown in (Figs.1 and 2). Large agglomerates of approximately 300 nm size composed of thin needle-like nanoHap particles of 10 nm thickness and 30 nm length were observed in the high Hap sample (Fig.1a). The particles with spherical morphology and dimensions 5-10 nm were found in the medium Hap (Fig.1b) samples, whereas very size-uniform spherical nanoparticles with a diameter around 5 nm are clearly visible in low Hap (Fig.1c).
Fig. 1. TEM image of dried nanohydroxyapatites prepared from high (a), medium (b) and low (c) concentrated solutions.

Fig. 2. HRTEM image of dried nanohydroxyapatites prepared from high (a), medium (b) and low (c) concentrated solutions, electron diffractogram from high Hap sample.
The substructures of individual nanoHap particles prepared from calcium α-D-heptagluconate solutions with different concentration are shown in Fig.2. In the HRTEM image of the high Hap sample (Fig.2a), larger well-ordered clusters about 5-10 nm dimensions surrounded with amorphous calcium phosphate phase were found. Similar substructure was observed in medium Hap samples (Fig.2b) but dimensions of well-ordered hydroxyapatite clusters did not exceed 5 nm. The number of ordered hydroxyapatite clusters was significantly raised in low Hap samples (Fig.2c) and simultaneously the dimension of clusters falls down under 5 nm. Note that volume fractions of ordered and amorphous regions were approximately the same. The electron diffractogram of high Hap sample verifies the presence of hydroxyapatite in clusters.

Figure 3 shows the XRD spectra of dried (105°C) and annealed (1000°C) Hap samples. The reflection from the (002) Hap plane was chosen for calculation of the crystallinity size using the Scherrer equation. The calculated crystallinity sizes were very close - 16 nm, 15 nm and 14 nm for high, medium and low Hap samples. Note, the Scherrer method for calculation of crystallinity size is not fully appropriate for crystallinity size determination in hydroxyapatite samples but in the case of measured XRD patterns (without sufficiently distinguished lines at wide interval of 2θ) of synthesized nanoHap, where the high fraction of amorphous disordered region and very fine ordered clusters were observed in Hap particles – the more accurate Williamson-Hall [17] method could not to be used. After annealing of the samples, both the crystalline Hap (JCPDS 24-0033) and β-TCP (JCPDS 09-0169) phases were found. The amount of β-TCP increased from low to high Hap samples but total amounts of both individual phases were high and comparable in all samples.

The FTIR spectra of dried and annealed Hap samples are shown in Fig.4. It is evident that samples have almost the same spectrum. In the IR spectra of dried Hap samples a broad band at ~3443 cm⁻¹ and one at ~1638 cm⁻¹ correspond to the physisorbed water on the Hap powders’ surfaces [18]. The peaks at ~3566 cm⁻¹ and ~632 cm⁻¹ characterize the stretching vibrations and librational mode of Hap OH groups [19]. The bands at ~1095 and ~1033 cm⁻¹ are typical for (ν₃) antisymmetric vibrations of PO₄³⁻ groups. Symmetric (ν₁) P-O stretching vibrations of PO₄³⁻ group are located at ~963 cm⁻¹, O-P-O bending (ν₄) vibrations at ~603 and ~564 cm⁻¹.

Fig.3. XRD patterns of dried (D) and annealed (A) Hap samples. (H)-hydroxyapatite, (β)-TCP.
Fig. 4. FTIR spectrum of dried: high (b), medium (d), low (f) and annealed high (a), medium (c), low (e) Hap samples.

Besides the absorption bands from vibrations of the CO$_3^{2-}$ group (see detail in Fig. 4), ($\nu_3$) and ($\nu_2$) vibrations were observed at 1560, 1542, 1489, 1455, 1422 and 875 cm$^{-1}$ respectively. From detailed analysis of carbonate bands, it results that the AB type of carbonated calcium deficient Hap was formed after 7 days of aging. However, after the annealing of dried samples at 1000°C, the carbonate bands completely disappeared from the Hap spectra because of the thermal decomposition of carbonates. The intensity of the peak at (~3572 cm$^{-1}$) from OH stretching vibrations of Hap was significantly higher in annealed samples because of recrystallization of the lattice. In accordance with XRD results, the peaks, which characterize both major phases – hydroxyapatite and β-TCP – were found in spectra. The shoulder at (~1119 cm$^{-1}$) and new bands at (~973 and ~947 cm$^{-1}$) can be assigned to ($\nu_3$) anti-symmetric and ($\nu_1$) symmetric P–O stretching vibrations of PO$_4^{3-}$ groups of β-TCP [20]. The peak at (~556 cm$^{-1}$) represents ($\nu_4$) anti-symmetric O-P-O bending vibrations for β-TCP. Note that the Ca/P mole ratio equals to 1.64 in all samples.

The calcium deficient nanohydroxyapatite is created by precipitation from the calcium α-D-heptaglucuronate solutions regardless of reactant concentration. The Hap particle size increased with the starting solution concentration because the high number of nuclei and a small Hap crystallites are created during precipitation from high concentrated solutions (high Hap samples were prepared even from supersaturated calcium α-D-heptaglucuronate solution), which have a large specific surface and high surface activity. This causes consequently their gradually recrystallization, growth particles during ageing and the presence of significantly larger well-ordered clusters in substructure what verified results TEM observations. In the case of Hap samples prepared from low concentrated reactant solutions, the number of critical nuclei created during precipitation was much lower and as a result of longer precipitation time (the same rates of reactant solution adding), precipitates reached the supercritical size and served as substrates for the nucleation of other Hap particles. This fact confirms the existence of the high number fine well-ordered clusters in low Hap particles substructure.

CONCLUSIONS

The nanocrystalline calcium deficient hydroxyapatites with a high fraction of amorphous phase and high number of well-ordered clusters in particles were prepared by
the precipitation from calcium α-D-heptagluconate solutions. The reactant concentration affected the particle size and morphology of the precipitated nanoHap. Biphasic mixtures with very close contents of Hap and β-TCP were found after annealing at 1000°C, regardless of the starting reactant concentration.

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