Original Article

Bioactivity Evaluation of Synthetic Nanocrystalline Hydroxyapatite

Mohammad Hossein Fathi*, Vajihesadat Mortazavi**, Seyed Iman Roohani Esfahani***

ABSTRACT

***Background*:** Bone regeneration in the defects around oral implants using substitutes may improve long-term prognosis of the implant. Hydroxyapatite (HA) is a good candidate for bone substitutes due to its similarity to bone minerals. Nanostructured hydroxyapatite is also expected to have better bioactivity than coarser crystals. The aim of this work was to synthesize and evaluate the bioactivity of HA.

***Methods*:** Nanocrystalline HA was synthesized via mechanical activation method. Fourier transform infrared spectroscopy (FTIR) was utilized to identify the functional groups of the prepared HA. Transmission electron microscopy (TEM) technique was utilized to evaluate the shape and size of prepared HA powder. The synthesized powder was soaked in stimulated body fluid (SBF) medium for various periods of time in order to evaluate its bioactivity. The changes of the pH of SBF medium were measured. Atomic absorption analysis (AAS) was used to determine the dissolution of calcium ion in the SBF environment and scanning electron microscopy (SEM) was utilized to evaluate the surface morphology of nanocrystalline HA powder after immersion in SBF.

***Results*:** The prepared HA powder had nano-scale morphological structure with the mean crystallite size of 29 nm in diameter and bone-like composition. The ionic dissolution rate of prepared nanocrystalline HA was higher than that in conventional HA and was similar to that of biological apatite of bone. High bioactivity of prepared nanocrystalline HA powder due to the formation of apatite on its surface was observed.

***Conclusions*:** Prepared nanocrystalline HA could be more useful for treatment of oral bone defects in comparison with conventional HA, and could be more effective as a bone replacement material to promote bone formation.

***Keyword*:** Bone substitutes, hydroxyapatite, nano-material, nanostructured material.

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Introduction

It is well known that bioactive materials can integrate well with living bone tissues by spontaneously forming a biologically active bone-like apatite layer on their surfaces.[1-4] Hydroxyapatite (HA) is the main mineral constituent of teeth and bones. HA ceramics do not exhibit any cytotoxic effects. They show excellent biocompatibility with hard tissues and with skin and muscle tissues. Moreover, HA can directly bond to the bone.[5] Multiple techniques have been used for preparation of HA powders, as reviewed in several works.[6] Two main ways for preparation of HA powders are wet methods and solid state reactions.[6,7] In the last five years, mechanosynthesis has been widely studied as an alternative technique to produce HA. Carbonate substituted HA[8,9] and nonsubstituted HA have been successfully produced following several mechanochemical synthesis routes.[10-14] Depending upon the technique, materials with various morphology, stoichiometry, and level of crystallinity can be obtained. Solid-state reactions usually give a stoichiometric and well-crystallized product.[5] Nowadays, the technique is used in a large range of commercial products. Moreover, most of these ap-

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plications are in the metallic domain. It is possible to improve the properties of HA ceramics by controlling important parameters of powder precursors such as particle size, particle distribution and agglomeration.[15] Nanocrystalline HA powders exhibit greater surface area and are expected to have better bioactivity than coarser crystals.[16,17] Osteoconductivity, solubility, sinterability and mechanical reliability of the HA can be promoted by controlling its particle size and structural morphology in the order of nanoscale.[15,18] Bone regeneration in the defects around oral implants by means of substitutes such as hydroxyapatite may improve the long term prognosis of the implant. HA is a good candidate for bone substitutes due to its chemical and structural similarity to bone minerals. Keeping the above points in mind, the present study was aimed to produce and to enhance the bioactivity of nanocrystalline HA by controlling its crystallite size and composition and to evaluate its bioactivity in simulated body fluid.

Materials and Methods

Synthesis of nanocrystalline HA powder

The present research was an experimental study. In this *in vitro* evaluation, nanocrystalline HA was synthesized by the mechanical activation method using a high energy planetary ball mill (Fritsch Pulverisette).[5] The starting materials were commercially available: dicalcium phosphate dihydrate ($CaHPO_4.2H_2O$, Merck > 98%, average size: 10 micron) and calcium carbonate ($CaCO_3$, Merck > 98%, average size: 5 micron). Appropriate amounts of the two starting materials were mixed together at a molar ratio of 3:2. The powder mixture was then loaded into a hardened steel bowl, together with a stainless-steel ball 20 mm in diameter. Mechanical activation reactions were performed in a high energy planetary ball mill at a rotating speed of 530 rpm. The mass ratio of balls to reactants was 20, whereby the overall ball mass was equal to 160 g. The time of mechanical activation by the planetary ball mill was selected equal to 2, 4, 6, 8, 9, 12, 16, 20, and 40 hours. HA powder in micron scale (Merck 2196, Darmstadt, Germany) was used as conventional HA for comparison of the bioactivity.

Characterization procedure

The phase composition of prepared HA powders was analyzed by X-ray diffraction (XRD) technique (X-ray diffractometer, Philips Xpert) using a CuKα radiation generated at 40 kV and 100 mA. Fourier transform infrared (FTIR) spectroscopy, (FT-IR, Bomem, MB100) was used to identify the functional groups of the prepared HA and to compare synthesized HA with bone apatite. The crystallite size of milled powder was determined by using the x-ray diffraction pattern and Williamson-Hall approach.[19]

$$\beta \cos\theta = \frac{k\lambda}{D} + 2A\sqrt{\langle \varepsilon^2 \rangle} \sin\theta \qquad (1)$$

where θ is the Bragg diffraction angle, D is the crystallite size, $\sqrt{\langle \varepsilon^2 \rangle}$ is average strain, λ is the wavelength of the used radiation, β is the diffraction peak width at half-maximum intensity, K is the Scherrer constant (0.91) and A is a coefficient which depends on the distribution of strain. Crystallite size D could be determined from the intersection of Williamson-hall line at sinθ = 0. The diffraction peak at 25.9° (2θ) corresponding to the (002) plane with (222) and (201) Miller planes family were chosen for calculation of the crystalline size. Transmission electron microscopy (TEM) technique (Philips CM 200 FEG: Eindhoven, The Netherlands) was utilized to evaluate the shape and size of prepared HA.

In vitro bioactivity evaluation

The *in vitro* bioactivity evaluation of synthesized HA powder was performed in a stimulated body fluid (SBF) media of pH 7.4 at a ratio of 1 mg/ml in a water bath at 37°C. The changes in the pH of SBF medium were measured at pre-determined time intervals using a pH meter. The dissolution amount of calcium ions in the SBF medium was determined by atomic absorption spectrometer. Scanning electron microscopy (SEM) was used to identify the apatite formation on surface of the samples and to evaluate the surface morphology of the samples after immersion in SBF medium.

Results

Phase and composition analysis

Figure 1 shows the XRD patterns of the powder mixtures of $CaHPO_4.2H_2O$ and $CaCO_3$ that were subjected to mechanical alloying for various periods of time (in the range of 2 to 40 hours). It could be seen that after 12 hours of mechanical activation process, HA could be formed. Figure 2 Shows Fourier transform infrared (FTIR) spectroscopy of nanocrystalline HA synthesized by 12

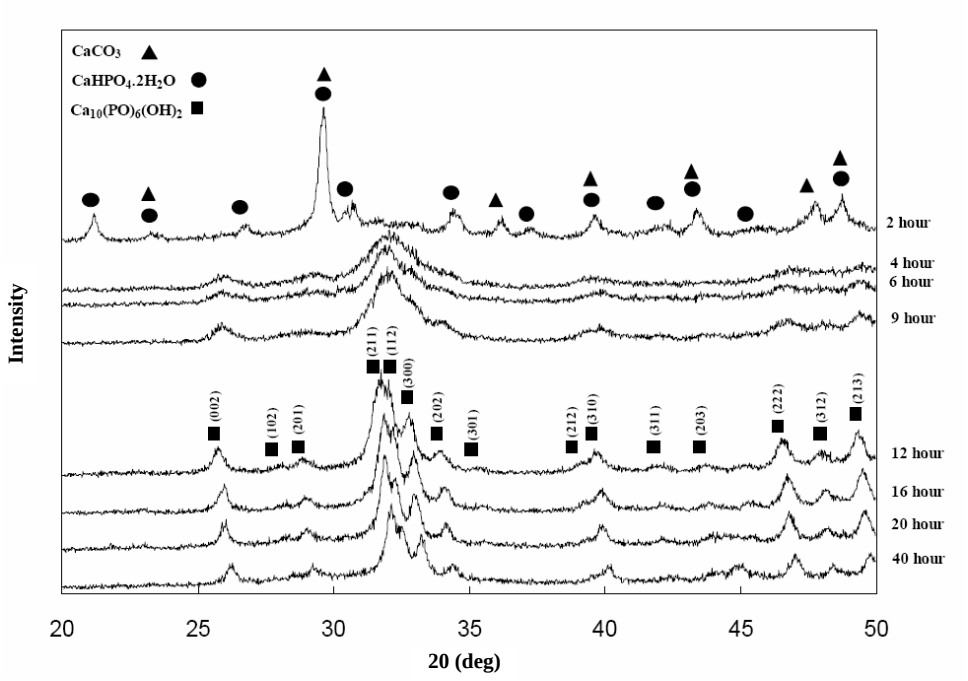

Figure 1. XRD patterns of the powder mixtures of $CaHPO_4.2H_2O$ and $CaCO_3$ after mechanical activation for various periods of time.

hours of mechanical alloying and also biological apatite of bone indicating that HA was synthesized similar to biological apatite of bone.

Figure 3 shows a TEM image of 12 hour-milled initial powders. It could be seen that nano-scale HA could be prepared after 12 hours of milling.

Bioactivity evaluation

The SEM micrographs of the surfaces of the immersed HA powder after soaking in SBF for various periods of time are shown in figure 4. Tiny agglomerated bone-like apatite particles could be formed on the surface of the HA powders soaked for 2 days. Figure 5 shows the rate of the releasing of calcium ions from the nanocrystalline HA into SBF indicating that the pattern of calcium releasing of nanocrystalline HA was similar to biological apatite. Figure 6 shows a graph of pH versus time, which illustrates the resorbability of nanocrystalline HA.

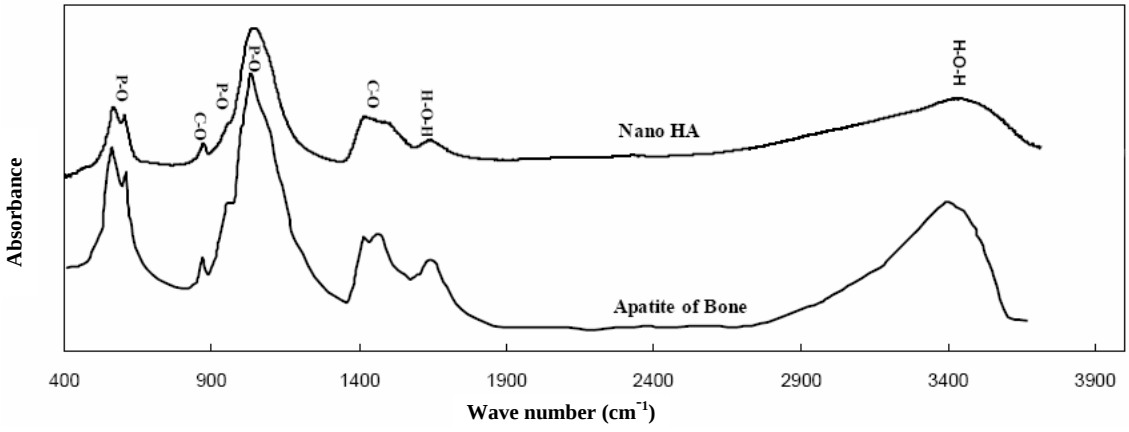

Figure 2. FTIR spectrum of prepared nanocrystalline HA and biological bone apatite.

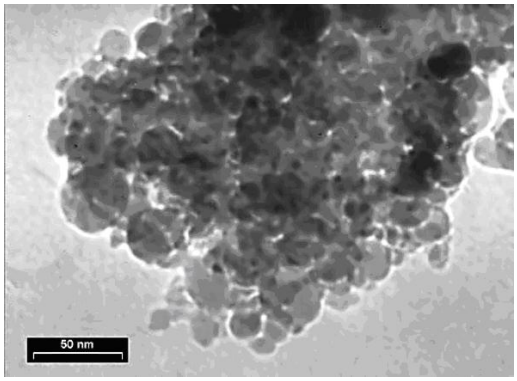

Figure 3. Transmission electron microscopy (TEM) micrograph of the prepared nanocrystalline HA powder obtained after 12 hours of milling.

Discussion

As it could be observed in figure 1, the 2 hour-milled powder showed only $CaCO_3$ and $CaHPO_4.2H_2O$ phases. By increasing the time of milling and mechanical activation process, the XRD peaks of the powders became broader and their peak intensities decreased. This trend continued up to 12 hours. At this stage, no elemental $CaCO_3$ and $CaHPO_4.2H_2O$ peaks could be observed, and according to JCPDS number 09-0432, the only present phase was HA. Further mechanical activation led to the formation and growth of nanocrystalline HA as the result of the mechanochemical reaction. However, no additional increasing in intensity was observed after 12 hours of milling, suggesting that the synthesis reaction was completed after 12 hours. The average crystallite size of produced HA after 12 hours of milling time was about 29 nm according to Williamson-hall approach.[19] It could be identified by the TEM image (Figure 3) that prepared HA powder after 12 hours of milling had almost a mean crystallite size similar to the determined size obtained by Williamson-Hall calculation. FTIR spectrum of nanocrystalline HA (Figure 2), showed all characteristic peaks of pure HA and additionally the characteristic peak of CO_3^{2-} group that appeared at 873, 1454 and 1769 cm^{-1}. Biological apatite of bone was also substituted with carbonate ions and had a similar FTIR pattern[20] to prepared nanocrystalline HA. Figure 4 shows that tiny agglomerated

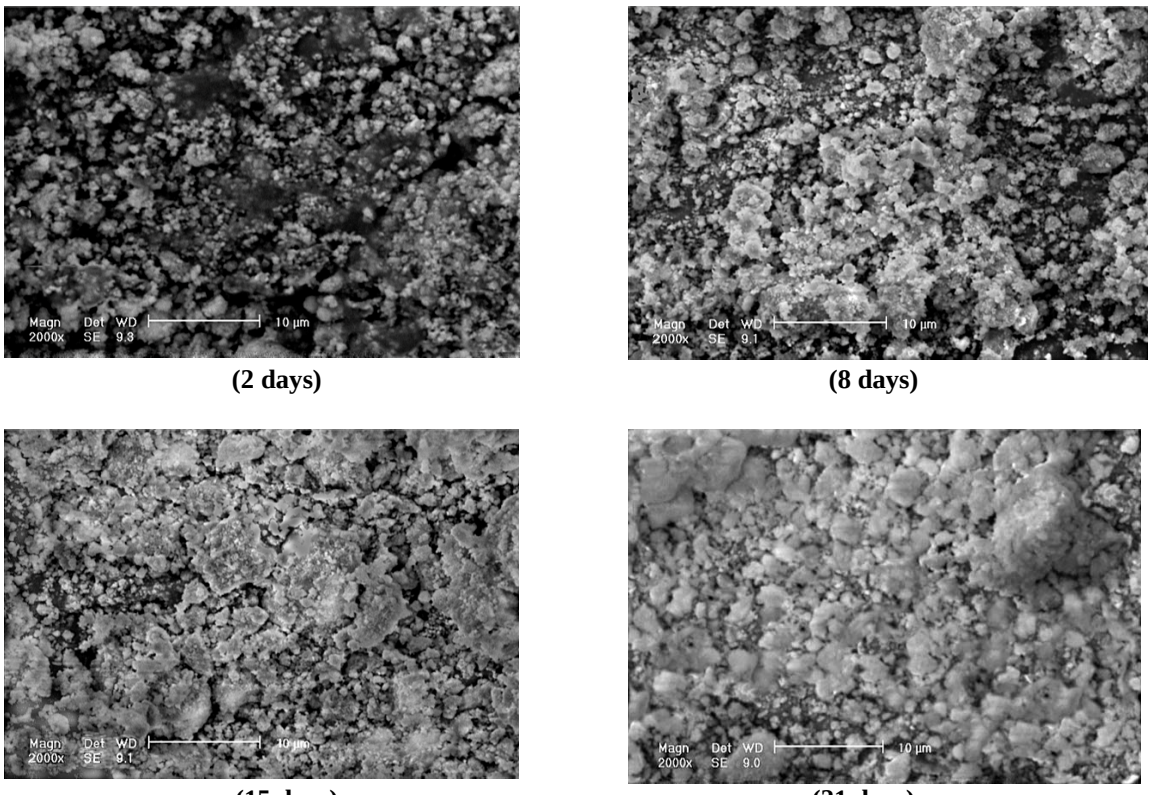

Figure 4. SEM micrographs of the surfaces of prepared nanocrystalline HA powders after soaking in SBF for various periods of time.

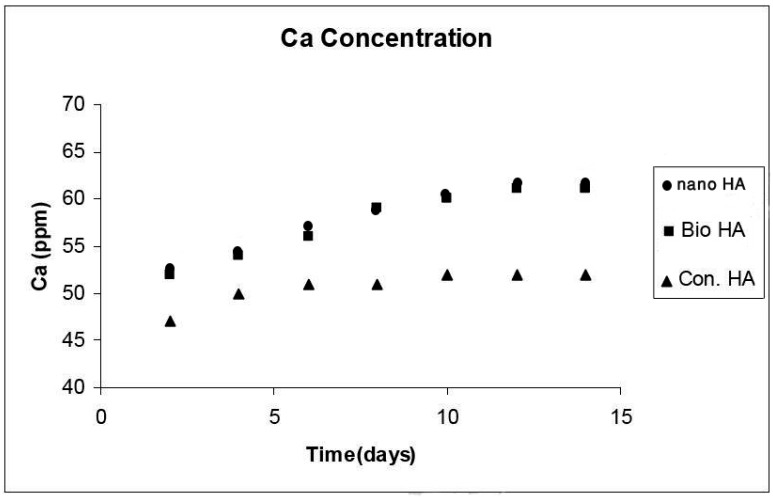

Figure 5. Release of calcium ions from the prepared nanocrystalline HA (nano-HA), biological apatite (Bio HA), and conventional HA (Con. HA) in simulated body fluid.

bone-like apatite particles could be formed on the surface of the HA powders soaked for 2 days. The number and the size of these agglomerated particles increased with increasing soaking times. Identification and evaluation of apatite formation on the surface of a material in SBF is useful for predicting the *in vivo* bone bioactivity of the material, not only qualitatively but also quantitatively.[19-22] The results indicated that the synthesized nanocrystalline HA powder showed the high bioactivity in SBF solution. The amount of the precipitations on the surfaces of nanocrystalline HA powder was interestingly more than the precipitations that were observed on the surfaces of micron crystallite size HA at the same condition in other research.[23]

The release of calcium ions from the nanocrystalline HA into SBF was quantitatively estimated to support its *in vitro* bioresorbability (Figure 5). The results showed that more calcium ions were released from the nanocrystalline HA in comparison with conventional HA. The amount of calcium release from the prepared nanocrystalline HA corroborated well with the calcium release pattern of biological apatite reported by other researchers.[21] The ionic dissolution rate of nanocrystalline HA is much similar to that of natural bone mineral *in vitro* in comparison with conventional HA. As solubility is highly sensitive to the structural and chemical compositions of the apatite samples, the crystallite size is an essential key factor for

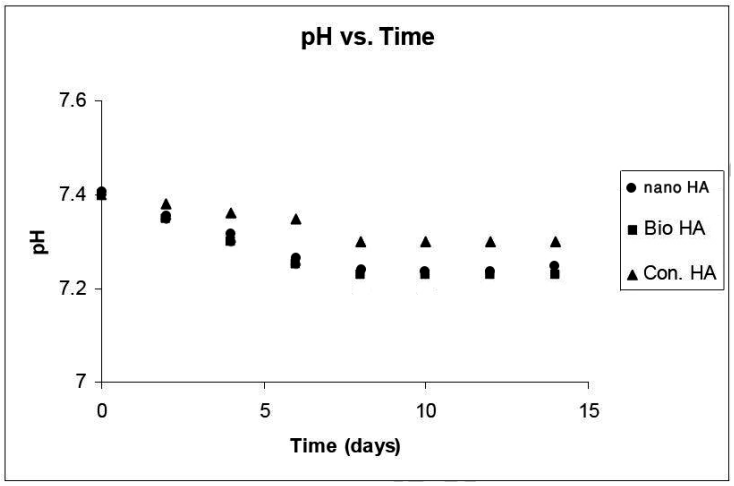

Figure 6. pH changes of SBF solution versus time due to biodegradation of prepared nanocrystalline HA (nano-HA), biological apatite (Bio HA), and conventional HA (Con. HA).

in vitro behavior of HA. In this manner, resorbability of HA could be promoted by engineering the crystallite size to nano-submicron level. The pH of the conventional HA was found to be of unvarying trend, as it was not resorbed in the medium,[21] which indicates its physiological stability during the period of study. The nanocrystalline HA showed drastic changes in the pH (Figure 6), suggesting that it dissolves much faster than the conventional HA. The pH value depends on solubility or resorbability of the HA. As the pH decreases, the solubility increases. Accordingly, it is clear from the graph that the rate of bioresorbability of the prepared nanocrystalline HA is higher than that in conventional HA and is similar to that in biological apatite of bone.[21] The solubility of HA could be altered depending on clinical needs by controlling its crystallite size. The synthesized nanocrystalline HA had amazing functional properties due to its crystallite size and *in vitro* properties similar to biological apatite, which would have a great impact on implant-cell interaction in a body environment. The bioresorbability of synthesized nanocrystalline HA powder was also similar to that in biological apatite when compared with another study.[21] Nanocrystalline HA possesses exceptional biocompatibility and bioactivity properties with respect to bone cells and tissues, probably due to its similarity with the hard tissues of the body.[24-27] Nanocrystalline HA is able to improve the contact reaction and the stability at the artificial/natural bone interface for medical applications.[21] Control of the crystallite size is a suitable parameter for tailoring behavior of the HA powders in dental and orthopedic applications. It was concluded that the prepared nanocrystalline HA could be more useful for treatment of oral bone defects in comparison with conventional HA and could be more effective as a bone replacement material to promote bone formation.

Conclusions

Nanocrystalline HA was obtained by 12 hours of mechanical activation. The prepared nanocrystalline HA powder exhibited an average particle size of 29 nm. *In vitro* bioresorbability and bioactivity of the nanocrystalline HA depend on its crystallite size. The prepared nanocrystalline HA powder showed high bioactivity similar to that in biological apatite and higher bioactivity in comparison with conventional HA. Prepared nanocrystalline HA might be more useful for treatment of oral bone defects in comparison with conventional HA, and might be more effective as a bone replacement material to promote bone formation.

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