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Hydroxyapatite nanoparticles: A review of preparation methodologies

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ABSTRACT: *Hydroxyapatite (HA) has been widely used as a biocompatible ceramic in many areas of medicine, but mainly for contact with bone tissue, due to its resemblance to mineral bone. In mammals, the skeleton presents a carbonated and partially substituted apatite, based on nanocrystal aggregates, and associated with collagen, building up 3-D structures present in various bone tissue conformations like trabecular or cancellous bone. There has been growing interest in developing bioactive synthetic ceramics that could closely mimic natural apatite characteristics. This review presents some of the most well known forms of obtaining, by precipitation methods, nanophased HA. Some traditional and more recent developments vis-à-vis the possible HA nanoparticles applications are discussed.(Journal of Applied Biomaterials & Biomechanics 2004; 2: 74-80)*

KEY WORDS: *Hydroxyapatite, Nanoparticles, Precipitation methods, Nanophase HA applications*

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INTRODUCTION

Nanosized hydroxyapatite (HA) is the main component of mineral bone. Living bone constantly undergoes a coupled resorptive-formative process known as bone remodeling. The process involves simultaneous bone removal and replacement through the respective activities of osteoblasts and osteoclasts, with the accompanying vascular supply and a network of canaliculi and lacunae.

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. To date, calcium phosphate biomaterials have been widely used clinically in the form of powders, granules, dense and porous blocks and various composites. Calcium phosphate materials form the main mineral part of calcified tissues. However, calcium phosphate presence in bone is in the form of nanometer-sized needle-like crystals of approximately 5-20 nm width by 60 nm length, with a poorly crystallized non-stoichiometric apatite phase containing CO_3^{2-}, Na^+, F^- and other ions in a collagen fiber matrix. Current research deals with new HA formulations aiming at better and more effective biomedical applications, producing this material with properties closer to those of living bone, such as nanosized and monolithic structures.

Compared to conventional ceramic formulations, nanophase HA properties such as surface grain size, pore size, wettability, etc, could control protein interactions (for example, adsorption, configuration and bioactivity); therefore, modulating subsequent enhanced osteoblast adhesion and long-term functionality. Webster et al (1-3) discovered that these enhanced osteoblast functions are proliferation, alkaline phosphatase synthesis and calcium containing mineral deposition. Nanometer grain size topography and surface wettability are nanoceramic material properties that not only promote increased selective vitronectin adsorption (a protein that mediates osteoblast adhesion) but also affect conformations that enhance osteoblast functions.

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NANOPHASE HA PREPARATION METHODS

There are several methods of preparing HA crystals reported in the literature, including wet chemical deposition, biomimetic deposition, sol-gel and electrodeposition.

1) An HA nanocrystal suspension can be prepared by a wet chemical precipitation reaction following the reaction proposed by Yagai and Aoki as indicated by Bouyer et al (4).

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18H_2O \quad [1]$$

The shape, size and specific surface area of the HA nanoparticles obtained by this method are very sensitive to the reactant addition rate, and to the reaction temperature. The reactant addition rate determines the purity of the synthesized HA and is linked strongly to the pH obtained at the end of the synthesis, and to the suspension stabilization. The reaction temperature determines whether the crystals are monocrystalline or polycrystalline. HA nanoparticles synthesized at low temperature (T<60 °C) are monocrystalline. A transition temperature (T=60 °C) can be defined as a limit for the monocrystalline HA nanocrystal synthesis, above this critical temperature nanocrystals become polycrystalline.

Another possibility, following the method proposed by Jarcho et al (5) is the wet chemical reaction of calcium nitrate ($Ca(NO_3)_2.4H_2O$), with ammonium hydroxide ($(NH_4)_2HPO_4$) the grain size controlled by changing the time and the temperature of HA precipitation (2, 3). Specifically, to obtain grain sizes <100 nm the solution requires stirring at room temperature for 24 hr.

Yubao et al (6, 7) prepared nanograde calcium phosphate needle-like crystals (Fig. 1) from wet synthesized Ca/P precipitates using $Ca(NO_3)_2.4H_2O$ and $(NH_4)_2HPO_4$ according to a method similar to Jarcho et al (5). The $(NH_4)_2HPO_4$ aqueous solution was added to a $Ca(NO_3)_2.4H_2O$ aqueous solution, with pH values between 10 and 12 and the reaction performed at room temperature. The as-prepared precipitates were put into an autoclave and hydrothermally treated at 140 °C and 0.3 Mpa for 2 hr. These crystals with a Ca/P ratio between 1.5 and 1.67 demonstrated a poorly crystallized apatite structure at room temperature and a biphasic (HA+β-TCP) structure after sintering at 1100 °C. Morphologically, these crystals were rod-like and 23 x 91 nm^2. Rod-like pure HA (Ca/P=1.67) crystals, with similar dimensions, can also be prepared using the same method, but with hydrothermal treatment

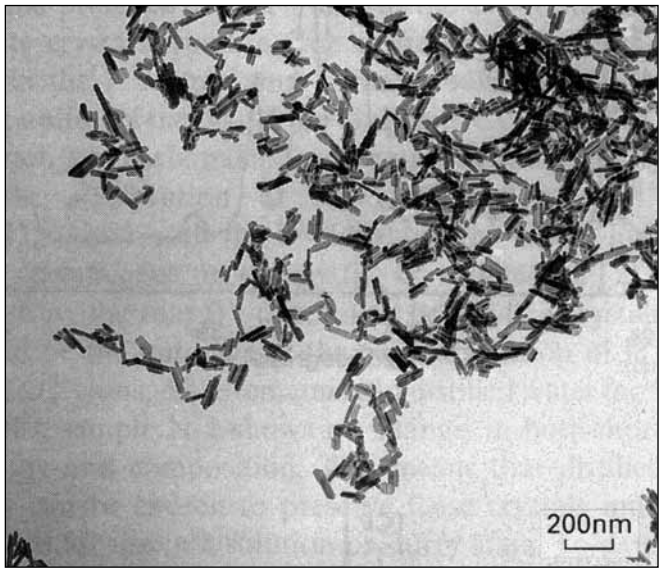

Fig. 1 - *TEM image of HA needle-like crystals obtained by wet chemical precipitation. Reprinted from: Yubao L, de Groot K, de Wijn J, Klein CPAT, Meer SVD. Morphology and composition of nanograde calcium phosphate needle-like crystals formed by simple hydrothermal treatment. J Mater Sci Mater Med 1994; 5: 326-31.*

at 200 °C and 2 Mpa for 10 hr. Therefore, the Ca/P ratio of the precipitates improves (in terms of similarity to HA) with an increase in hydrothermal pressure or temperature.

Janackovic et al (8) modified the method based on the homogeneous precipitation technique using the following reaction:

$$Ca(EDTA)^{2-} + 3/5\ HPO_4^{2-} + 2/5H_2O \rightarrow 1/10Ca_{10}(PO_4)_6(OH)_2 + HEDTA^{3-} + 1/5OH^- \quad [2]$$

The modification developed consisted of the addition of urea for precipitation instead of NaOH, which led to more homogenous monetite precipitation and further transformation to HA due to pH changes because of urea hydrolysis. The synthesis temperature varied between 125 and 160 °C. During urea hydrolysis, the CO_3^{2-} ions were released and were incorporated in the HA crystal structure as in human bones. Increasing temperature, precursor concentration or reaction time results in preferential crystal growth along the 001 plane.

In addition, HA nanoparticles can be prepared by wet precipitation under stirring at room temperature and pH=10. H_3PO_4 is added to $Ca(OH)_2$ and $C_3H_6O_3$ until it becomes Ca/P=1.67. Crystallization starts after NH_4OH addition. Crystal growth was allowed for 24 hr and sinterization performed at 1100 °C for 1 hr. The final product was nanosized

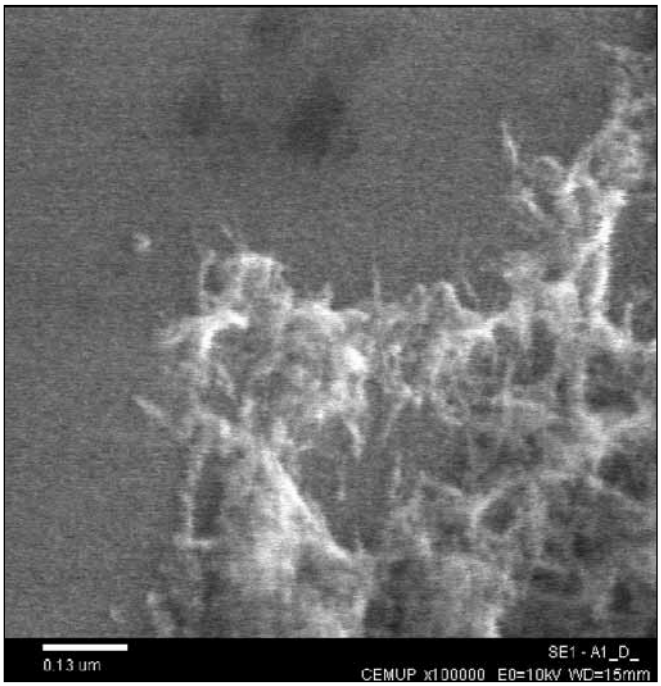

Fig. 2 - ***SEM image of aggregates of HA nanoparticles obtained by wet chemical precipitation. Reprinted from: Manuel CM, Ferraz MP, Monteiro FJ. Synthesis of hydroxyapatite and tricalcium phosphate nanoparticles- preliminary studies. Key Engineering Materials 2003; 240-242: 555-8.***

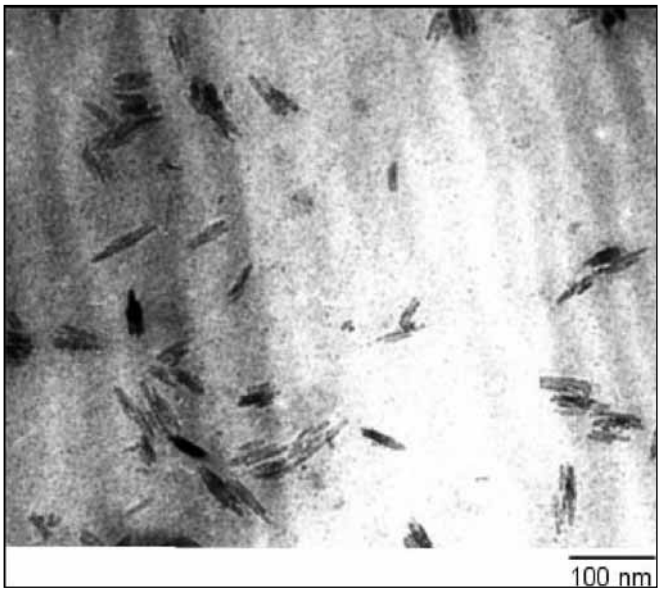

Fig. 3 - ***TEM image of discrete HA nanocrystals obtained by wet chemical precipitation (ibid).***

and homogenous HA (Figs. 2, 3) (9, 10).
2) Metastable synthetic body fluids (modified SBF), with an inorganic salt composition similar to that of human blood plasma, incubate and facilitate the spontaneous nucleation and growth of a nanosized, carbonated and "bone-like" calcium HA at physiological pH and temperature. This biomimetic HA powder can be obtained from calcium nitrate tetrahydrate and diammonium hydrogen phosphate salts dissolved in SBF at 37 °C and with a pH=7.4 by a chemical precipitation technique (11). SBF prepared according to the chemical analysis of human body fluid, with ion concentrations nearly equal to the inorganic constituent of human blood plasma, were first used by Kokubo (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 coated with a poorly crystallized calcium-deficient and carbonate-containing apatite similar to bone apatite (13). Metastable SBF has been proven to incubate and facilitate the spontaneous generation and growth of a carbonated and "bone-like" calcium apatite on immersed silica or titania gels, bioglass and titanium samples at physiological pH and temperatures (14-18). The presence of this carbonated apatite layer formed by a biomimetic process on several materials was proven to promote *in vitro* cell differentiation in a mineralizing chondrocyte cell culture system (19) and induce osteogenic cell differentiation and subsequent bone matrix apposition, which allows a strong bond to bone (20).
Nanosized and chemically homogeneous HA were synthesized at physiological and biomimetic conditions, 37 °C and pH=7.4, by a chemical precipitation technique from a modified SBF containing calcium nitrate tetrahydrate and dissolved diammonium hydrogen phosphate salts. In addition, these powders contained traces of other inorganic ions provided and incorporated by the SBF. The average grain sizes of this powder after sintering remained sub-micron. When heated at 1200 °C for 6 hr, they were found to reach approximately 96% densification (11).
It is possible to evenly coat porous implants with nanosized carbonated HA and other Ca/P compounds biomimetically, which consists of mimicking the bone mineralization process by immersing implants in SBF (21-23). The nature of the Ca/P coating, via its microstructure, its dissolution rate and its specific interactions with body fluids, can influence the osteogenecity of the coating (24) as well as the bone remodeling process (25).
Using the biomimetic coating method, in contrast to other coating techniques, biologically active agents can be added to the supersaturated solutions and gradually be co-precipitated with the calcium phosphate crystals, forming a layer on the metal im-

plants (26, 27). This creates the possibility of also uniformly incorporating an antibiotic within the biomimetic coating and releasing it at a controlled rate; therefore, preventing local post-surgical infection (28).
The wide range of potential biomimetic applications explains the large number of recently published studies on this topic (21, 23, 24, 28-32).
3) Low-temperature formation and apatitic crystal fusion are the main contributions to the sol-gel process, in comparison to conventional methods. In fact, temperatures >1000 °C are usually required to sinter the fine apatite crystals prepared from wet precipitation, whereas temperatures several hundred degrees lower are needed to densify sol-gel HA. The sol-gel method offers a molecular-level mixing of the calcium and phosphorus precursors, which is capable of improving the chemical homogeneity of the resulting HA to a significant extent. HA synthesis requires a correct 1.67 calcium to phosphorus molar ratio in the final product. A number of Ca and P precursor combinations were employed for sol-gel synthesis.
In addition to the difference in the precursors' chemical activity, the temperature required to form the apatitic structure depends largely on the chemical nature of the precursors. Gross et al (33) and Masuda et al (34) used calcium diethoxide ($Ca(OEt)_2$) and triethyl phosphate ($PO(OEt)_3$) to form pure HA phase at temperatures >600 °C. They found that ageing times >24 hr were critical for the solution system to stabilize, in order that a monophasic HA could be produced, otherwise there were large weight losses during pyrolysis and undesirable phases, such as CaO observed (35). Jillavenkatesa et al (36) synthesized a mixture of HA and CaO at 775 °C using calcium acetate ($Ca(C_2H_3O_2)_2$) and triethyl phosphate as precursors. This process required further hydrochloric acid leaching to eliminate CaO, leading to pure HA phase formation. Brendel et al (37) obtained HA at temperatures as low as 400 °C using calcium nitrate ($Ca(NO_3)_2.4H_2O$) and phenyldichlorophosphite ($C_6H_5PCl_2$) as precursors. However, the resulting HA had low purity and poor crystallinity. A further increase in the synthesis temperature to 900 °C resulted in a pure, well-crystallized HA phase.
Takahashi et al (38) developed a gel route using calcium nitrate and phosphonoacetic acid ($HOOCCH_2PO(OH)_2$) in an aqueous solution and obtained a pure HA powder at 700 °C. HA crystallinity increased with temperatures up to 1100 °C. Haddow et al (39) used calcium acetate together with a number of phosphorus precursors, i.e. phosphoric acid (H_3PO_4), phosphorus pentoxide (P_2O_5) and triethyl phosphite for HA coating applications. They found that the films prepared from triethyl phosphite and calcium acetate showed the best wetting characteristic and the temperature required to form an apatitic phase was >600 °C.
Recently, a novel, low-temperature water-based sol-gel process for HA synthesis was developed using triethyl phosphite and calcium nitrate as P and Ca precursors, respectively (40). A two-step procedure was employed to synthesize HA. The phosphite was initially hydrolyzed with water for 24 hr, followed by the addition of an aqueous nitrate solution. Subsequently, the amorphous gel transformed into a well-crystallized apatite at relatively low temperatures (300-400 °C). The calcinated gels showed a nanoscale microstructure, with grains of 20-50 nm diameter. Through appropriate heat treatment between 300 and 400 °C, the apatite prepared exhibited a nanoscale, low-crystallinity, carbonated apatitic structure, resembling that of human bone apatite (41).
4) Ultrafine-grained, nanophase HA coatings can be synthesized by electrocrystallization from dilute electrolytes $[Ca^{2+}]=6.1x10^{-4}M$, $[PO4^{3-}]=3.6x10^{-4}M$ at physiological pH (Fig. 4). At these low supersaturations, HA is precipitated without a precursor phase formation (42). The electrolytes used for the electrodeposition of calcium phosphate coatings were prepared using $Ca(NO_3)_2$ and $NH_4H_2PO_4$ dissolved in de-ionized water. $NaNO_3$ was used to improve the electrolytes' ionic strength. The electrolytes' pH was adjusted in order that in all cases the electrolytes were saturated with respect to HA (values

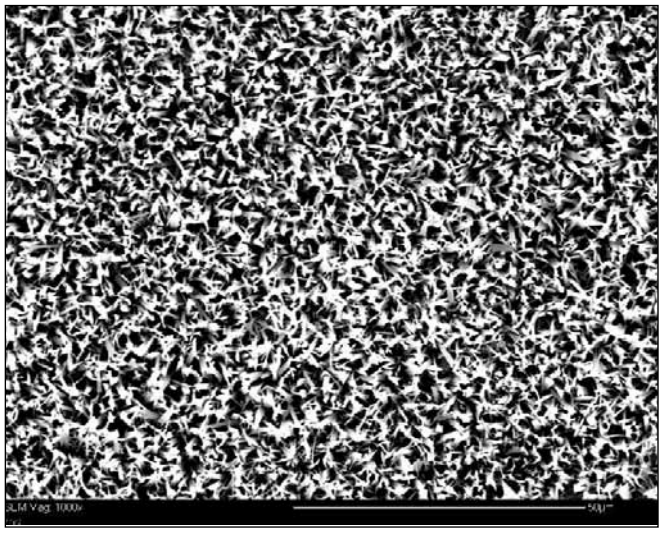

Fig. 4 - ***SEM of the calcium phosphate coating obtained by electrocrystallization (11). Reprinted from: Shirkhanzadeh M. Direct formation of nanophase hydroxyapatite cathodically polarised electrodes. J Mater Sci Mater Med 1998; 9: 67-72.***

determined from the solubility isotherm for HA in the ternary system $Ca(OH)_2$-H_3PO_4-H_2O). Calcium phosphate electrodeposition was carried out for 2 hr at 85 °C. Deposits precipitated from the acidic electrolytes consisted of plate-like crystals of 4-6 µm and deviated markedly from stoichiometric HA. At very low concentrations of calcium and phosphate ions and physiological pH, nanophase HA is deposited directly on the cathodes.
Manso et al (43), tested growth induced by constant anodic voltages (2-4 V) in an alkaline electrolyte. The deposition method of HA coatings resulted from the modification of a precipitation reaction (44). Essentially, the changes consisted of the use of an electrochemical cell and a set of different final concentrations. They obtained a well-crystallized nanosized HA coating, through electrical activation of spontaneous deposition.

Main applications for nanophase hydroxyapatite

Although it is not yet commercially available as a competitive material with respect to other forms of HA, nanosized HA is currently used for several applications, which are either in advanced research states or undergoing development with considerable commercial opportunities.
Du et al (45) studied the tissue response of nano-HA-collagen implants in marrow cavities and concluded that nanoparticles allowed for a quicker implant surface turnover. The process of implant resorption and bone substitution was similar to bone remodeling.
Muller-Mai et al (46) tested nanoapatite and nanoapatite / organic implants *in vivo*. From their results, it was seen that both materials were suitable for bone replacement and for drug release such as antibiotics, growth factors or other substances. In addition, the organic component can be used to control physical properties in the bone implantation bed.
Among other applications, the following can be considered.
1) HA coating based on sol-gel technology (41) or electrodeposition (42, 43), allowing for the formation of thin adherent films, which do not severely affect the substrate morphology and topography.
2) Composite preparations with other materials like chitosan (47, 48), collagen (45, 49-53) and other polymers (54), able to reinforce the matrix while promoting osteoconduction; therefore, providing scaffolding properties required in tissue engineering applications.
3) Nanosized HA can be used in drug delivery systems like intestinal delivery of insulin (55), or other drugs such as antibiotics (56).
4) Further examples studied include its use in genetic therapy for certain types of tumors (56).

CONCLUSIONS

Therefore it can be concluded that new HA based materials are certainly among the most promising challenges in bioactive ceramics for the near future, and consequently, the research effort put in their development will continue to increase.

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