vol 3 (2) pag. 43-57

REVIEW ARTICLE

Nanoparticles of hydroxyapatite: preparation, characterization and cellular approach - An Overview

Alis Yovana Pataquiva-Mateus,[1] *María Pia Ferraz,[2,4] Fernando Jorge Monteiro[2,3]

[1] Universidad Jorge Tadeo Lozano, Departamento de Ingeniería, Carrera 4 Nº 22-61 Bogotá, Colombia. [2] INEB—Instituto de Engenharia Biomédica, Laboratório de Biomateriais, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal. [3] Universidade do Porto, Faculdade de Engenharia, Departamento de Engenharia Metalúrgica e Materiais, Porto, Portugal. [4] Universidade Fernando Pessoa, Praça 9 de Abril 349, 4249-004 Porto, Portugal. * Autor para correspondencia: alisy.pataquivam@utadeo.edu.co

Nanopartículas de hidroxiapatita: preparación, caracterización y aproximación celular – Revisión

Abstract

Nanostructure syntheses have been applied to derive hydroxyapatite in nanometric scale for orthopaedic and dental implant applications due to its biocompatibility and similarity to natural apatite characteristics, becoming to a preferred material compared to hydroxyapatite in micro scale. This overview presents some of the most studied processes of obtaining nanophased hydroxyapatite, enriched with results from our group and additional and interesting information about cell-nanohydroxyapatite performance *in vitro* experiments.

Keywords: nanoparticles, ceramics, hidroxyapatite, bone engineering, dental implant.

Editor: Hernández J.

Citation: Pataquiva-Mateus AY, Ferraz MP, Monteiro FJ. (2013) Nanoparticles of hydroxyapatite: preparation, characterisation and cellular approach - An Overview. Revista Mutis. vol. 3 (2) pag. 43-57

Received: September 2, 2013; **Accepted:** November 29, 2013; **Published on line:** December 15, 2013

Competing Interests: The authors have no conflict of interest

Resumen

Las síntesis de nanoestructuras se han aplicado para obtener hidroxiapatita a escala nanométrica para aplicaciones de implantes ortopédicos y dentales debido a su biocompatibilidad y a la similitud de las características con apatitas naturales, convirtiéndose en un material preferido para la comparación con hidroxiapatita a microescala. Este resumen presenta algunos de los procesos más estudiados para obtener hidroxiapatita en nanofase, enriquecido con los resultados de nuestro grupo y la información adicional e interesante sobre el rendimiento celular de nanohidroxiapatita *in vitro*.

Palabras clave: nanopartículas, cerámicos, hidroxiapatita, ingeniería de hueso, implante dental.

Introduction

When bone is lost due to injury and/or illness, the defects are filled with natural or artificial bone grafts, but with infections and bioaffinity as collateral problems, respectively. Hence, research has recently being fo-

cused towards artificial materials, which might present biological properties similar to those of bone and that might replace natural bone by grating.

In recent years, apatite and others calcium orthophosphates have shown substantial interest and importance due to their chemical similarity with the natural calcium phosphate. Calcium orthophosphates are salts of the tribasic phosphoric acid, H_3PO_4, and this forms three groups of compounds that contain $H_2PO_4^-$, HPO_4^{2-} or PO_4^{3-} ions (Elliot, 1994). The first group are compounds formed only under acidic conditions and so they are not found normally in biological systems. The compounds of second and third groups are found in the mineral component of bones and teeth, as well in some pathological calcifications.

Among apatites, Hydroxyapatite (HA), ($Ca_{10}(PO)_6(OH)_2$) is known to be the major inorganic constituent of bone, corresponding to almost 70% in dry weight, of bones hard tissues (Hench, 1991, Klein *et al.*, 1994, Lacefield, 1999, Lavernia & Shoenuna, 1991). The term apatite was firstly coined by Werner in 1786, which is derived from the Greek word *apataw* (Elliot, 1994) that means "to deceive" because frequently the minerals, belonging to this group, were misleading due to similar appearances with valuable stones.

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 (Bayraktar & Tas, 1999). There are several reports dealing with the precipitation of HA, modifying stoichiometries from aqueous solutions in order to obtain different Ca/P ratios (Akao, Aoki & Kato, 1981, Akao *et al*., 1982, Chair, Heughebaert, & Heughebaert, 1995, Ishikawa, Ducheyne, & Radin, 1993, Nordstrom & Karlsson, 1990, Osaka *et al*., 1991b, Rouer *et al*., 1993). Since Jarcho and colleagues in 1976 until now, many studies about HA preparation have been reporting the synthesis of crystals of HA in the nanometric, sub-micrometric and micrometric size ranges (Dhont, Verbeeck & De Mayer, 1996, López-Mancipe, Gómez-Morales & Rodríguez-Clemente, 1998, Suchanek *et al*., 1995, Wang *et al*., 1995, Zong & Gonsalves, 1997). However, the various parameters that affect size, shape, and crystallinity of nanoscale HA (1-100 nm) still need further study.

Hydroxyapatite Properties

In general, calcium phosphates are all white solids, unless doped with a coloured ion and most are sparingly soluble in water, although some as HA are quite insoluble, but all dissolve in acids (Elliot, 1994). HA has a relatively high thermal stability and phase purity ever after high temperature sintering (1000-1300°C) (Bayraktar & Tas, 1999).

HA has been proposed as substitute for defective bones or teeth. It is known to be biocompatible, bioactive, i.e. ability to form a direct chemical bond with surrounding tissues, osteoconductive, non-toxic, non-inflammatory and non-immunogenic agent (Hench, 1991, Jarcho, 1981, Ogiso, 1998).

HA appears to form a direct chemical bond with hard tissues (Piattelli & Trisi, 1994, Uehara, Takaoka, & Ito, 2004) and on implantation of HA particles of porous blocks in bone, new lamellar cancellous bone forms within 4 to 8 weeks (Spanos, Deimede & Koutsoukos, 2002). HA ceramics have a flexural strength comparable to human bone. However, the elastic modulus and compressive strength of HA ceramics are higher than those of human bone, while the fracture toughness is significantly higher for human bone than for HA ceramics (Tanaka, Hirata & Yoshinaka, 2003).

Nowadays, HA is widely applied as a coating artificial joint components used in orthopaedics and dental implants and hold a great potential as a biomaterial for bone implantation due to its bioactivity, dissolution range, and resorption properties close to those of natural bones (Rodriguez-Lorenzo & Vallet-Regi, 2000). According to the literature, conventional HA has been widely applied in the forms of powder, dense or porous blocks (Hench, 1991, Murugan & Panduranga Rao, 2002).

Currently, significant research effort has been dedicated to developing inorganic nanosize crystalline materials because of their new possibilities deriving from size and their potential in biology, electronics, optics, transport, and information technology (Combes & Rey, 2002, Gorbunoff, 1984a, b, c, Hench, 1991, 1998, Kawasaki, Niikura & Kobayashi, 1990b, a, Suzuki, Ishikawa & Miyake, 1984, Zahouily *et al*., 2003). It is believed that nanostructured calcium phosphate ceramics can improve the sintering kinetics due to a higher surface area and subsequently a significantly increase in the

mechanical properties (Bose & Saha, 2003). Furthermore, most likely the nanoscale sizes of the particles will provide an increase in specific surface area, which in turn can cause an increase in the binding capacity of macromolecules such as DNA (Kumta, Sfeir & Lee, 2005) and thus might dramatically affect the biological outcome.

Differences in shrinkage between the agglomerates are also responsible by the occurrence of small cracks in sintered HA (Ruys *et al*., 1995). Therefore, synthesis of agglomerate-free or softly agglomerated nanostructured HA may be an important step towards achieving improved mechanical properties in dense nanostructures (Banerjee, Bandyopadhyay & Bose, 2007).

Recently, HA prepared at nano level has been playing an increasingly significant role in various biomedical applications owing to its unique functional properties of high surface area to volume ratio and its ultrafine structure similar to biological apatite, which is having a great impact on cell–biomaterial interactions (Webster, Siegel & Bizios, 2000, Zhu *et al*., 2006). This type of HA has been considered mainly in the form of particulate for the treatment of periodontal osseous defects and alveolar ridge augmentation because of its easy fabrication, handling and close surface contact with the surrounding tissue (Doi & Horiguchi, 1996).

The attractive advantages described above justify a further review of HA nanocrystals synthesis, characterisation and its behaviour in contact with cells.

NanoHA applications

Needle-like HA particles are suitable for preparing filters for ion exchange between cations (Suzuki, Ishikawa & Miyake, 1984) or for protein adsorption (Kawasaki, Niikura & Kobayashi, 1990b, Tiselius, Hjertén & Levin, 1956). In particular, colloidal HA has been used as adsorbents for chromatography to separate proteins and as catalysts for chemical reactions such as the Michael-type addition and methane oxidation (Akazawa *et al*., 1999, Sugiyama *et al*., 1999, Zahouily *et al*., 2003).

NanoHA preparation has a considerable academic and commercial interest for bone replacement and coatings on metallic joint prostheses. HA occurs in ammoniated superphosphates, and in calcareous soils as a reaction product of monocalcium phosphate monohydrate ($Ca(H_2PO_4)_2.H_2O$) or brushite. In addition, nanofiber-shaped HA is a promising candidate material for reinforcing ceramic or polymer matrices used in the biomedical applications (Wang *et al*., 2006).

Magnetic nanoparticles have been developed to such an extent that the functional properties have been specially tailored for biological applications such as cell separation, drug delivery, a a contrast agent for magnetic resonance imaging (MRI), and as a heat mediator for hyperthermia (Wu *et al*., 2007).

Obtaining non-agglomerated nanosized bioceramic particles of high-surface area is interesting for many applications including injectable/controlled setting bone cements, high strength porous/non-porous synthetic bone grafts, the reinforcing phase in nano-composites that attempt to mimic the complex structure and superior mechanical properties of bone (Phillips *et al*., 2003); and as potentially useful materials for a number of biomedical applications including scaffolds for tissue engineering and as carriers for non-viral gene delivery (Kumta, Sfeir & Lee, 2005).

NanoHA Preparation Methods

Considering the numerous applications of calcium phosphate compounds in biomedical fields, several nanoHA synthesis techniques and variations have been developed.

HA nanostructured can be produced from dry and wet reactions, being this one the most widely studied. Wet chemical reactions to form calcium phosphate materials can be generally divided in two types of processes in terms of reagents concentrations: non-homogeneous and homogeneous processes (Bezzi *et al*., 2003). A dropwise addition of a donor reagent of phosphorous into a calcium hydroxide solution or salt slurry is involved in the non-homogeneous process; while, in the second one, calcium and phosphorous salts are solubilised in water under a strict pH control. In both cases a long ageing time is required to let the precipitation reactions be concluded, in order to obtain uniformity of a single ion concentration during the precipitation phase (Bezzi *et al*., 2003).

Previous work mainly placed emphasis upon controlling the stoichiometry of the product, while, with the development of nanotechnologies, considerable

effort is now focused on controlling the morphology and size (Hirai, Hodono & Komasawa, 2000, Sarda, Heughebaert & Lebugle, 1999, Walsh & Mann, 1996, Yuan, Liu & Peng, 2002) due to studies that have shown that many clinical capabilities of HA highly depend on its morphology and size (Hing, Best & Bonfield, 1999).

Mechanochemical processing

The principle on which mechano-chemical synthesis during the reduction of powders particles is founded, relates to the energy provided by the impaction that mill balls exert on powder particles constrained towards the internal wall of the jar. Moreover, about temperature it can rise in the collision region may vary from few degrees to few hundred degrees (Soni, 2001). This method takes advantage of the perturbation of surface-bonded species by pressure to enhance thermodynamic and kinetic reactions between solids (Gutman, 1997). During milling, the energy transmitted to crystalline particles results in a dislocation cell structure that develops into random nanosized particles increasing the milling time (McCormick & Froes, 1998). Spherical particles with 10-50 nm in size of carbonated-substituted HA were produced from phosphate di-hydrogen ammonium and calcium carbonate as precursors after 6h of milling (Coreño *et al*., 2005).

Sol-gel route

Sol-gel technique is considered as an elective method for the preparation of highly pure powder due to the possibility of a strict control of the process parameters. This particular synthesis process induces better chemical homogeneity and its product is characterized by nano-size dimensions of the primary particles, which improve the contact reaction and the stability at the artificial/natural bone interface (Bezzi *et al*., 2003). In addition, sol-gel is characterized by being a low temperature process because there is a highly reactive powder without any degradation phenomena occurring during sintering. Nevertheless, hydrolysis of phosphates and expensive raw materials are the limitations to this technique (Jillavenkatesa & Condrate, 1998).

In other studies (Chu *et al*., 2002), citric acid ($C_6H_8O_7$) was added as a chelating agent to avoid agglomeration of HA particles in the presence of calcium nitrate and trimethyl phosphate as precursors in a sol-gel processing. Primary and secondary particles produced have 50 nm and 100-200 nm, respectively, where these last are aggregates of the first ones. Through the addition of citric acid, the substitution of organic macromolecule for hydrate molecule can lower the reaction speed making favourable the HA gel particles uniformly distributed by the organic constituent and difficult for them to aggregate and grow.

Biomimetic process

The biomimetic process is inspired in the biomineralization of bone, which involves the deposition of apatitic crystals on a matrix rich in collagen fibrils, which are the structural macromolecules that create the scaffold within which the biological mineral is formed. Moreover, the biological matrix contains acidic macromolecules that are rich in negatively charged groups, mainly carboxylates but also sulfates and phosphates. These macromolecules are believed to control nucleation, polymorphism, growth, chemical composition, shape, dimensions, orientation, and texture of the crystals (Lowenstam & Weiner, 1989). The presence of the acidic macromolecules usually inhibits the nucleation and growth of calcium salts in aqueous solution, whereas they promote calcification when adsorbed on a substrate (Huer *et al*., 1992). Several synthetic macromolecules and polyelectrolytes are known to influence nucleation and growth of calcium salts, resembling the role that biological macromolecules play *in vivo*. Even after extensive investigations related to biomineralization, many intermediate stages are still unclear. This complex biological process means crystallization of apatite, formed biologically, onto the surface of either already formed bone or biological macromolecules, being possible this process in presence of hundreds of biological compounds having some influence in the mineralization process (Dorozhkina & Dorozhkin, 2002).

For that purpose, several authors have been used synthetic body fluids (SBF) (Bayraktar & Tas, 1999, Bigi *et al*., 2000, Dorozhkina & Dorozhkin, 2002, Ilmori *et al*., 2005, Kokubo, 1990, Landi *et al*., 2006, Muller & Muller, 2006, Noris-Suárez *et al*., 2007, Oyane *et al*., 2003) that have similarities with the chemical analysis of human body fluid, with ion concentrations nearly equal to those of the inorganic constituents of hu-

man blood plasma. SBF were firstly used by Kokubo *et al.* (Kokubo, 1990) to prove the similarity between *in vitro* and *in vivo* behaviour of certain glass-ceramic compositions (See Table 1).

Table 1. Ion concentrations of SBF solution and "human plasma".

Ion	Human plasma (mM)	Kokubo *et al*., 1990 (Kokubo 1990) (mM)	Bayraktar & Tas, 1999 (Bigi *et al*. 2000) (mM)	Bigi *et al*., 2000 (Bayraktar & Tas 1999) (mM)
Na^+	142.0	142.0	142.0	141.5
Cl^-	147.8	125.0	103.0	124.5
HCO_3^-	4.2	27.0	27.0	27.0
K+	5.0	5.0	5.0	5.0
Mg_2^-	1.5	1.5	1.5	1.5
Ca_2^+	2.5	2.5	2.5	2.5
HPO_4^{2-}	1.0	1.0	1.0	1.0
SO_4^{2-}	0.5	0.5	0.5	0.5

SBF is known as a meta-stable buffer (Ohtsuki, Kokubo & Yamamuro, 1992), however, its preparation must be very careful because a small variance in either the preparation steps or the storage temperatures, has a radical effect on the phase purity and the kinetics of the HA precipitation processes.

Two routes were proposed (Bayraktar & Tas, 1999) using di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) and calcium nitrate solutions ($Ca(NO_3)_2.4H_20$) as phosphate and calcium ions sources, respectively. The syntheses were carried out at 37°C, to avoid SBF degradation reaching HA powder with Ca/P ratio of 1.67. In addition, Mg- and CO_3^{2-} substituted HA was performed obtaining particles of 30-40 nm in size (Landi *et al*., 2006) and it was found that pH is a primary variable in the mineralization process (Dorozhkina & Dorozhkin, 2002).

Hydrothermal method

There are not many studies on the hydrothermal synthesis of nanoHA. The source of calcium phosphates can be either organic or inorganic. The purity and mechanical property of the products from the organic source are comparatively inferior to those from the inorganic one, i.e. calcium phosphates extracted from bone are likely to retain traces of products are equally biocompatible (Jinawath, Pongkao & Yoshimura, 2002). In general, the high purity chemical reagents are much more expensive than bone ash, so it is interesting to prepare calcium phosphates compounds with minimal impurities from bone or its related substances. Low temperature (≤ 200°C) hydrothermal treatment is recommended (Yoshimura, 1998) as an attractive inexpensive and environmentally friendly solution process with high purity products.

The mechanism of organization of organic surfaces; that can control the nucleation of inorganic materials by geometric, electrostatic, and stereochemical complementary between incipient nuclei and functionalized substrates; has been reviewed by several authors (Yan *et al*., 2001).

Emulsion technique

Microemulsion is a technique based on the use of two immiscible liquids such as water that has a high dielectric constant being dispersed under agitation in an oil phase, having low dielectric constant, both phases stabilized by an ampiphilic surface-active agent or surfactant in order to control interfacial tension. Microdroplets of water, formed when the aqueous phase is dispersed surrounded by a monolayer of surfactant molecules in the hydrocarbon phase, are stabilized in a non-aqueous phase by a surfactant acting as a microreactor or nanoreactor in which reactions are conducted (Bose & Saha, 2003). These self-assembling media of reverse micelles can be used in the synthesis of nanophase materials without excessive agglomeration, and with high surface area. Hence, nanoHA with spherical morphology can be produced by this method. It has been shown to be one of the few techniques, able to produce a particle size in the range of nanometers with minimum agglomeration (Pileni, 2003).

Nanocrystalline HA particles have been synthesized using a microemulsion technique (Bose & Saha,

2003) with Ca and P ions and NP-5 and NP-12 surfactant in cyclohexane organic solutions. It was shown that for HA nanopowders, their surface area, morphology and particle size were strongly affected by the composition of the microemulsion system, pH, aging time, temperature and metal ions concentration. The results of nanoHA particles characterisation showed surface areas around 130 m^2/g and particle sizes in the range of 30-50 nm with needlelike morphology, among others.

Sonoda and co-workers (Sonoda *et al*., 2002) have reported HA synthesis with small size and aspect ratio using pentaethyleneglycol dedecyl ether emulsion similar to a nonoemulsion. Lim *et al*. (Lim *et al*., 1996) have reported the synthesis of HA powders with a specific surface area between 42 and 80m^2/g and an average particle size between 0.53 and 1.15 µm with agglomerated morphology; and formation of nanocrystalline HA in nonionic surfactant emulsion where the specific surface area varied from 7-75 m^2/g depending on petroleum ether concentration. Lower average surface area (26 m^2/g) with high particle size (200 nm), including a strongly agglomerated morphology of 45 µm in size, was reported (Layrolle, Ito & Tateishi, 1998).

The reverse microemulsion technique (Guo *et al*., 2005) appears as a nanoHA technique where the particle length depends directly of hydrophile-lipophile balance (HLB) value, reaching particles from 21 up to 93 nm but without any uniform shape.

For HA nanofibers, the hydrothermal microemulsion (Cao *et al*., 2004), regarding 1D structures, appears as a variation of emulsion technique and led to a substantial increase in the aspect ratio, a fairly satisfying uniformity in diameter (50-120 nm), a well-crystallized product, and a shorter reaction time. The hydrothermal method has been proved to be an effective method to synthesize materials in nano dimensions. It was also was proved that, after the HA nucleation in CTAB reverse micelles, the surfactant head groups preferentially adsorb on surface planes parallel to the c axes of the nuclei, resulting in the formation and growth of anisometric particles. These tiny particles then aggregate in a directional manner though their c axes onto ultrahigh-aspect-ratio HA nanofibers (Cao *et al*., 2004).

Chemical precipitation

The most commonly used technique for the formation of HA is the precipitation, which involves wet chemical reactions between the calcium and phosphate precursors under controlled temperature and pH conditions (Aoki & Kato, 1975, Asada *et al*., 1987, Hattori, Iwadate & Kato, 1988, Jarcho *et al*., 1976, Osaka *et al*., 1991a). However, this method has some inherent disadvantages such as the difficulty to control of the pH value over 9 to avoid the formation of a Ca-deficient HA, which during sintering forms tricalcium phosphates (Bezzi *et al*., 2003). Chemical synthesis of HA powders in neutral and /or slightly acidic aqueous media is known to be a more complicated and difficult task (Ebrahimpour *et al*., 1993, Madsen & Thodvadarson, 1984).

In our own experiments, during titration was observed that viscosity decreased as a function of H_3PO_4 addition, and so at pH 10.5 the white solution obtained is visibly less viscous in comparison with the starting point at pH 13. These findings are in agreement with reported elsewhere (Bao, Senos & Almeida, 2002), where to a solution of HA different percentages of a polyacrylic acid dispersant (Dispex N40) were added, showing that for pH values between 9.5 and 11 the suspensions show a minimum in viscosity for the three concentrations of dispersant. These results indicated that the best condition for the stabilization of the HA suspensions is achieved for a pH around 10.5 (Bao, Senos & Almeida, 2002).

Recently, as a variation of chemical precipitation, surfactant-based template systems are being explored to synthesize nanosize materials and they are assumed to be very efficient templates for controlling particle size and shape (Pileni, 2003). Agglomeration can cause lower sintered density as well as crack-line voids during densification. High aspect ratio morphology in the starting powder can also cause poor packing, which results in exaggerated grain growth during sintering (Bose & Saha, 2003). Consequently, to produce sintered monolithic HA parts with desired mechanical properties, will be need to use agglomerate-free low aspect ratio fine Ha powders with high surface area.

Nanostructure processing of HA in the presence of ytria-stabilized zirconia by chemical precipitation and pressure-assisted sintering of these powders, has also been reported (Ahn *et al*., 2001).

Materials and Methods

Chemical precipitation was the processing route chosen for nanohydroxyapatite synthesis because it is a low cost, simple and widely studied process. Henceforth, experimental data is related to hydroxyapatite nanophased obtained from this process.

NanoHA synthesis

Aqueous solution of calcium hydroxide ($Ca(OH)_2$) and ortho-phosphoric acid (H_3PO_4, 85%), both of analytical grade, were used as starting reagents in a chemical precipitation for the preparation of HA nanoparticles according to the following procedure: firstly, 100 ml of an aqueous suspension of H_3PO_4 (0.6M) was very slowly added, drop by drop, to a 100 ml of an aqueous suspension of $Ca(OH)_2$ (1M) added with sodium dodecylsulfate (SDS) into the calcium solution while stirring vigorously for about 30 min at room temperature. Concentrated NaOH was added until a final pH of 10.5 was obtained. The white solution obtained was washed using de-ionized water and dried in oven at 80°C for 24 h.

NanoHA caracterisation

The characterisation methods listed below were performed without any further physical or thermal treatment of nanopowder.

A) Physical characterisation

Biodegradation study

In order to investigate the degradation behavior of nanoHA synthesized was carried out a comparison of biodegradation of three types of nanoHA, one of them was the commercial nanoHA (nH-1) and the other two were prepared by different routes: chemical precipitation (describe above, nHA-2) and hydrothermal process (nHA-3). Discs of nanoHA were incubated with solutions of phosphate buffered saline (PBS) and SBF during 1, 3, 7 and 15 days at 120 rpm and 37°C.

Inductively Coupled optical emission spectrometry (ICP-OES)

Three specimens of nanoHA obtained from chemical precipitation process describe above, were dissolved in conc. HCl, and afterwards were diluted in a 2% of HNO_3 solution to obtain concentrations in the range of 10 – 100 ppm for each element (Ca, P). Quantification was performed using an ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometer, Activa-S, Horiba Jobin Yvon, NJ). Atoms in the plasma emitted light to detect phosphorus and calcium at 213 nm and 317 nm, respectively. The intensity of the energy emitted at the chosen wavelength was proportional to the element concentration in the analyzed sample, and the values obtained were compared with standards in the range of concentration above mentioned.

Scanning electron microscopy analysis – SEM

The powder morphology was analyzed using digital imaging and scanning electron microscopy (SEM). Using a JEOL JFC-100 fine coat ion sputter device, samples were sputter coated with gold. Then they were studied with a JEOL JSM-6301F microscope at an accelerating voltage of 5 kV.

Transmission electron microscopy – TEM

The size and morphology of the nanoHA powder was determined using a Transmission Electron Microscope (TEM, Zeiss model EM 902A) at an accelerating voltage of 80kv. A nanoHA solution was prepared by dispersing 0.5mg of nanoHA in 1mL of ethanol. This was placed in an ultrasonic bath overnight, to ensure thorough dispersion. A drop of the solution, agitated for 1min in vortex, was placed on a Formvar carbon-coated copper 400 grid and left for 1 min. Excess liquid was removed with filter paper and the grid was allowed to dry for 10 min. TEM images were acquired and observed with Axion Camera Zeiss and AxionCs 40AC Zeiss, v 4.2.0.0 software.

B) Chemical characterisation

Infrared spectroscopy

Chemical characterisation was performed using Fourier transformed infrared (FTIR) spectroscopy. For this purpose, nanoHA was ground in an agate mortar and thoroughly mixed with spectroscopic grade KBr. 1-2 mg of nnaoHA powder sample was mixed with 200 mg of KBr powder. Pellets were analyzed using a PerkinElmer System 2000 spectrometer. FTIR was performed to determine the various functional groups in phosphates and carbonate substitution in the synthesized nanoHA powder.

X-ray Photoelectron Spectroscopy - XPS

XPS analysis was performed using VG Escalab 200R facility equipped with hemispheric detector with 5 channeltrons (energy steps of: 2-200 eV) X rays source with doubleMg/Al anode; pre-chamber with both temperature and atmosphere controlled on gun in pre-chamber and in main chamber (ISS) Electron gun for Auger spectroscopy

X-ray diffractometry – XRD

X-ray diffraction characterisation of nanoHA powder was conducted using a Panalytical X´Pert PRO alpha-1 with a RTMS X´Celerator detector. It used Ni-filtered Cu Kα radiation over the 2teta range of 10-90° at a scan rate of 2.4° /min and with a sampling interval of 0.002° at 40 mA and 45 kV.

C) *In vitro* performance

MG-63 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air, in α-MEM (GIBCO 22561-021), 10% fetal bovine serum (FBS), 1% fungizone and 0.5% gentamicin. For the subculture, the cell monolayer was washed twice with PBS and incubated with trypsin-EDTA solution (0.05% trypsin, 0.25%EDTA) for 5 min at 37°C to detach the cells. Cells were re-suspended in culture medium for re-seeding. There were synthesized porous and uniform microspheres (500 µm) based on nanoHA particles that were sterilized at 120°C for 20 min, placed on a 96 PS culture plate (14 mg of microspheres in each well, *c.a* 1cm^2 of surface area) and incubated with PBS overnight. Cells were then seeded on microspheres pre-conditioned with α-MEM with 10%FBS, 1% fungizone and 0.5% gentamicin. MG-63 cells were cultured (2×10^4 cells/cm^2) up to 7 days under controlled conditions (cells seeded on 96 TCPS plates) and on the surface of both type of microspheres. The control cultures and seeded microspheres were evaluated throughout the incubation time (7 days) for cytotoxicity (Neutral Red) and observed by confocal microscopy (CSLM) and scanning electron microscopy (SEM). Medium was substituted by fresh one every other day.

Cytotoxicity tests (Neutral Red)

Cytotoxicity of nanoHA-1 and nanoHA-2 microspheres was investigated on MG-63 osteoblast-like cells, which express a number of features characteristic of relatively immature osteoblasts (34) using Neutral Red assay. A 0.4% w/v solution of neutral red dye tested for cell cultures (Color Index 50040) in distilled water was prepared and stored at 4°C (stock solution). The working solution was prepared immediately before use by diluting the stock solution in complete medium to yield a final concentration of 50 µg/mL. Five replicates were performed for each type of microspheres. The negative control was microspheres in culture medium in PS 96 wells culture plates. The positive control was MG-63 in culture medium in 96 wells TCPS culture plates. After incubation for 1, 3, 5, and 7 days at 37°C, the supernatant was discarded, 0.150 mL of neutral red dye solution were added to the wells for 3 h at 37°C. The supernatant was removed, the wells washed twice with PBS and 0.1 mL of lysing solution (50% ethanol in 1% acetic acid) was added. The color intensity of each well was read at 540 nm with reference to 620nm, the optical densities of the replicates were averaged and the viability read in negative control was discounted.

Cell morphology (SEM and CLSM)

For SEM, microspheres-cells constructs were washed twice with PBS and fixed with 1.5% glutaraldehyde in 0.14% cacodylate solution for 30min at room temperature. They were dehydrated by suspending in successive ethanol solutions (50%, 70%, 80%, 90%, and 100%) for 10min in each solution. After dehydration, the samples were transferred to hexamethyldisilazan and air-dried at room temperature overnight. Finally, the microspheres-cells constructs were sputter coated with gold and examined under a JEOL JSM-6301F microscope at accelerating voltage of 5kV.

For CSLM, samples were washed twice with PBS, fixed in 4% v/v formaldehyde (methanol-free; Polyscience) for 15 min, permeabilized with 0.1% v/v Triton X-100 for 5 min, and incubated in 10 µg/mL bovine serum albumin and 100 mg/mL RNAse for 1h at room temperature. F-actin filaments were stained with Alexafluor-conjugated phalloidin (Molecular Probes) for 20 min at RT in dark, and nuclei were counterstained with 10 µg/mL propidium iodide (Sigma) for 15 min. Finally, samples were washed with PBS, mounted in Vectashield® and stored at 4°C in dark. CLSM images were acquired on a BioRad MRC 600 microscope.

Results

Biodegradation

The disintegration of nanoHA discs in both solutions (PBS and SBF), in crescent order after 15 days was nanoHA-3> nanoHA-2> nanoHA-1. All nanoHA samples increased their weight during the experience as was evidenced in SEM images (data not showed) where it was observed deposition of crystals (NaCl) and new nanoHA formation. This fact suggests that synthesized nanoHA were more biodegradable that commercial sample, obtaining an interest feature for a biomaterial used in bone tissue engineering.

ICP-OES

ICP accounts deserve special attention the value found for the loss of mass (20.7%) that indicates the presence of volatiles substances, which may be OH, H_2O or CO_2. As the hypothesis of CO_2 will be unlikely since is not a constituent of the raw materials. However, as the stoichiometric amount of OH in apatites rarely exceeds 5%, it is suggested that the sample contains an excess of H_2O, probably encapsulated in its structure. The value of Cl is found compatible with a chlorine-apatite. The values of CaO and P_2O_5 are relatively low for a natural apatite. However, if the analysis is recalculated, disregarding the value of water, following values are obtained CaO = 55% and P_2O_5 = 42%, which are perfectly compatible with a stoichiometric apatite.

SEM

Morphological results from SEM investigation are shown in Figure 1, where it is observed that the morphology of the HA nanocrystals presents a uniform needle-like shape.

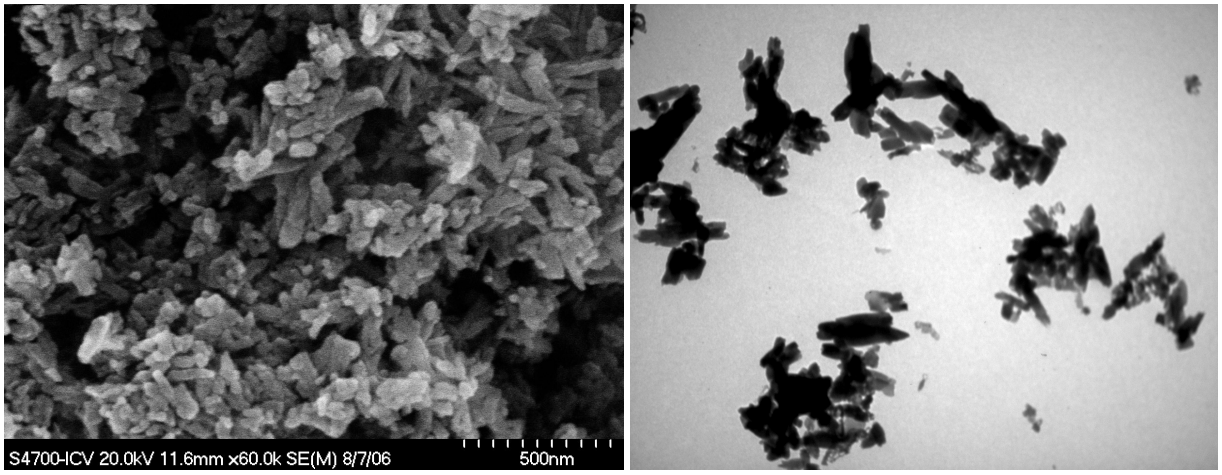

Figure 1. SEM (left) and TEM (right) micrographs. NanoHA particles have been studied using these methods to characterize morphologically these nanocrystals that are presented in agglomerates of few microns. Nanometric particles of HA exhibit in both micrographs bar and needle-like shapes with 80x20nm dimensions in mean. (SEM, scale bar=500nm; TEM, 12000x).

TEM

Morphology of nanoHA particles using Transmission Electron Microscopy is observed in the Figure 1. Dispersed crystals of nanoHA, due probably to the surfactant use with bar and needle-like shapes, are observed in a high magnification.

FTIR

FTIR results (data not showed) suggested the presence of bands characteristic of PO_4^{2-} at 471 (*v2*), 563 (*v4*), 961 (*v1*) and 1000 cm^{-1} (*v3*) (Panda *et al*., 2003); OH^- vibrational due to water at 602 cm^{-1} and occluded water at 1638 and 3431 cm^{-1}. It is important to point out that bands at 1422 cm^{-1} (*v3*, in-plane stretching band) and 872 cm^{-1} (out-of-plane stretching band) suggesting the presence of a carbonated nanoHA, which may have practical applications. It is well known that the presence of carbonate in calcium phosphate materials is an important factor contributing to their reactivity and to the *in vivo* integration of the implant (Ribeiro, Barrias & Barbosa, 2006). The bands at 1545,

1450 and 880 cm^{-1} were thought to be due to a small fraction of the total CO_3^{2-} ions replacing OH^- ions in the lattice (Elliot, 1994).

XPS

Results from XPS are presented in Table 2. It is noteworthy that the atomic ratio of material P/Ca or as well more current expressed Ca/P = 1,71 near to the natural apatite with a calcium / phosphorous ratio of 1,67. Besides, Table 1 presents P2p and Ca 2p 3/2 values similar to natural apatite, as well.

Table 2. Binding energies (eV) of core electrons and surface atomic ratios of apatite

Sample	P 2p	Ca $2p_{3/2}$	O 1s	C 1s	P/Ca atomic	CO_3^{2-}/Ca atomic
Apatite	133.2	347.2	531.0	289.7	0.585	0.106

XRD

A typical XRD profile of HA ceramic synthesized by calcium hydroxide and ortho-phosphoric acid to ceramic conversion has been shown in Figure 2. XRD analysis of the nanoHA powder indicated the presence of monophase non-crystalline compound.

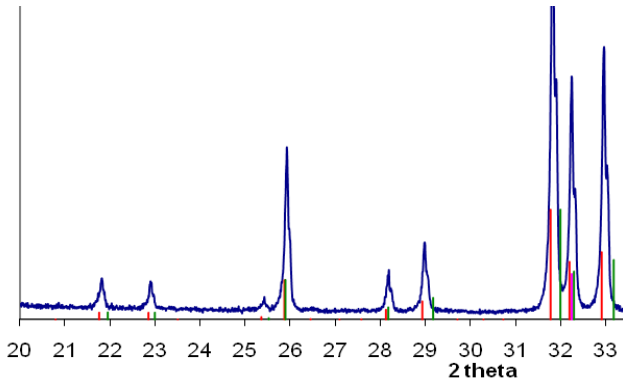

Figure 2. XRD spectra of nanoHA in powder. It is indicated that results (blue line) fit the most of peaks (vertical lines) that exhibits natural hydroxyaptite.

Biological Assessment

In the present work, the cytotoxicity tests of nanoHA it was assessed by the synthesis of microspheres based on nanoHA particles (Mateus *et al.*, 2008). Biological evaluation was performed using human MG-3 osteoblast-like cells line. Cells seeded on TCPS, used as a control, showed the same trend, but with a higher growth rate than microspheres. The control presented less number of viable cells in the first day probably due to the less available surface area compared to the microspheres. The nanoHA microspheres presented a significant number of viable until the end of the culture, and high growth rate as well.

SEM images of MG-63 osteoblast cells cultured for 7 days on the surface of nanoHA microspheres based on nanosized hydroxyapatite are presented in Figure 3. The surface of nanoHA microspheres is almost completely covered by layers of cuboidal cells which became denser along the entire time of culture, forming confluent monolayers. CLSM images in Figure 3 show nanoHA microspheres uniformly colonized by osteoblasts cells after 5 days of culture.

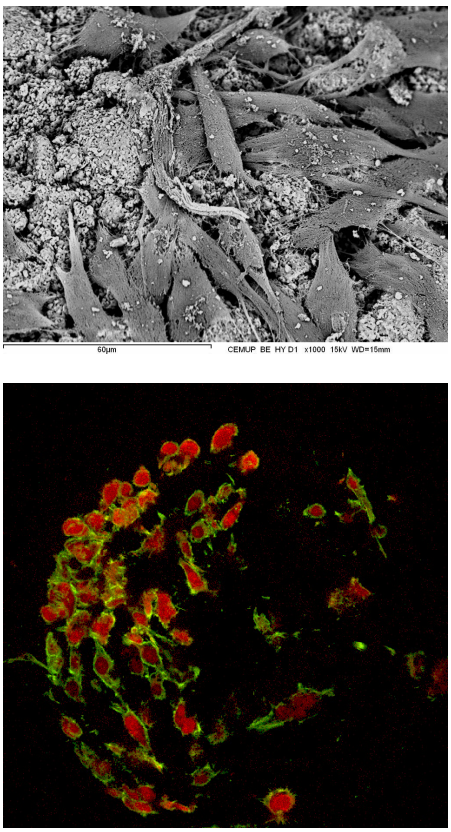

Figure 3. SEM (left) and Confocal (right) micrographs of seeded microspheres with MG-63 osteoblast cells after 7 days of culture. In the left, osteoblasts proliferation was observed on nanoHA microspheres means SEM using backscattered electron imaging. In the right, a CLSM image shows aligned osteoblasts proliferating on microsphere surface where nuclei is stained in red and cytoplasm stained in green.

Discussion

In general, the nanoHA synthesis using a number of methods; including mechano-chemical, sol-gel, biomineralization, chemical precipitation, emulsion techniques, batch hydrothermal processes, being all of these the most used methods to processing nanoHA; having several disadvantages such as requiring a very precise control over reaction conditions, expensive starting materials, large amounts of toxic organic solvents, or time consumption (Chaudhry *et al*., 2006). However, chemical precipitation is the most outstanding process due to its simplicity and good results without many sophisticated instruments, equipments or reagents.

NanoHA prepared by chemical precipitation was characterized using different method that has allowed verifying results about morphology, size, chemical composition and biological behavior. The use of different methods provides to researcher support for initial results and a better understanding about material from different points of views.

Synthesized nanoHA can be useful as nanocrystals, aggregates, powder, or as well conforming discs and microspheres, been a versatile material in bone engineering scaffolding with many action fields.

So far as we know, no review has revised extensively a vast number of preparations processes, characterization methods enriched with own data that exemplify the knowledge, as presented in this work.

Conclusions

Therefore it can be concluded that new nanoHA 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.

Being bone engineering one of the action fields where nanoHA play an important role, others such as pharmaceutical, cosmetics, catalysis and instrumentation, are industrial sectors benefited from the new technology applied in the nanoHA processing.

Therefore, it can be conclude that among bioactive ceramics, the hydroxyapatite and its processing into particles smaller than one micron have poured on this well-known ceramic, interesting new abilities in several areas. Specifically in biomedical engineering are brought new opportunities in scaffolding, drug delivery system and even DNA transportation.

Acknowledgments

A.Y. Pataquiva Mateus is grateful to the Portuguese Foundation for Science and Technology (FCT) for awarding her a scholarship (SFRH/BD/16616/2004). This work was performed under contract POCTI/FCB/41523/2001 (Gaucher) ou POCTI/SAU - BMA/56061/2004 (COLCPNA). The technical support of Rui Fernandes – IBMC (TEM), Miguel Angel Rodríguez – ICV/CSIC (SEM, XRD), Juan G. Fierro – ICP/CSIC (XPS), and Ana Lopes – INEB (FTIR).

References

Ahn, E., N. J. Gleason, A. Nakahira & Ying J. Y. (2001). Nanostructure processing of hydroxyapatite-based bioceramics. *Nano Letters,* 1 (3): p.149-153.

Akao, M., H. Aoki & K. Kato. (1981). *Journal of Material Science,* 16: p.809.

Akao, M., H. Aoki, K. Kato & A. Sato. (1982). *Journal of Material Science*, 17: p.343.

Akazawa, T., M. Kobayashi, M. Yoshida, K. Matsushima, H. Minoshima, H. Sugimura, T. Kanno & J. Horiuchi. (1999). Improved liquid chromatographic separation of different proteins by designing functional surfaces of cattle bone-originated apatite. *Journal of Chromatography A*, 862: p.217-220.

Aoki, H. & K. Kato. (1975). *Ceram Jpn,* 10: p.469.

Asada, K., K. Oukami, S. Nakamura & K. Takahashi. (1987). *Ceram Soc Jpn*, 95: p.703.

Banerjee, Ashis, Amit Bandyopadhyay & Susmita Bose. (2007). Hydroxyapatite nanopowders: synthesis, densification and cell-materials interaction. *Materials Science & Engineering C*, 27: p.729-735.

Bao, Y., M. R. Senos & M. Almeida. (2002). Rheological behavior of aqueous suspensions of hydroxyapatite (HAP). *Journal of Material Science: Materials in Medicine*, 13: p.639-643.

Bayraktar, D. & C. Tas. (1999). Chemical preparation of carbonated calcium hydroxyapatite powders at 37°C in urea-containing synthetic body fluids. *Journal of the European Ceramic Society*,19: p.2573-2579.

Bezzi, G., G. Celotti, E. Landi, T. M. G. La Torretta, I. Sopyan & A. Tampieri. (2003). A novel sol-gel technique for hydroxyapatite preparation. *Materials Chemistry and Physics*, 78: p.816-824.

Bigi, Adriana, Elisa Boanini, Silvia Panzavolta, & Norberto Roveri. (2000). Biomimetic growth of hydroxyapatite on gelatin films doped with sodium polyacrylate. *Biomacromolecules*,1: p.752-756.

Bose, S., & S. K. Saha. (2003). Synthesis and characterization of hydroxyapatite nanopowders by emulsion technique. *Chemistry of Materials*,15: p.4464-4469.

Cao, M., Y. Wang, C. Guo, Q. Yanjuan & C. Hu. (2004). Preparation of ultrahigh-aspect-ratio hydroxyapatite nanofibers in reverse micelles under hydrothermal conditions. *Langmuir*, 20: p.4784-4786.

Chair, H., J. Heughebaert & M. H. Heughebaert. (1995). *Journal of Material Chemistry,* 5: p.895.

Chaudhry, Aqif A., Saba Haque, Suela Kellici, Paulin Boldrin, Ihtesham Rehman, Fazal A. Khalid & J. A. Darr. (2006). Instant nano-hydroxyapatite: a continuous and rapid hydrothermal synthesis. *Chemical Communications,* p.2286-2288.

Chu, C. L., P. H. Lin, Y. S. Dong & D. Y. Guo. (2002). Influences of citric acid as a chelating reagent in the characteristics of nanophase hydroxyapatite powders fabricated by a sol-gel method. *Journal of Materials Science Letters*, 21: p.1793-1795.

Combes, C. & C. Rey. 2002. Adsorption of proteins and calcium phosphate materials bioactivity. *Biomaterials*, 23: p.2817-2823.

Coreño, J., O. Coreño, J. J. Cruz & C. Rodriguez. (2005). Mechanochemical synthesis of nanocrystallune carbonate-substituted hydroxyapatite. *Optical Materials*, 27: p.1281-1285.

Dhont, L., R. M. H. Verbeeck & E. A. P. De Mayer. (1996). *Journal of Material Science: Materials in Medicine*, 7: p.201.

Doi, Y. & T. Horiguchi. (1996). Formation of apatite–collagen complexes. *Journal of Biomedical Material Research: Part A*, 31: p.43-49.

Dorozhkina, Elena & Sergey Dorozhkin. (2002). Surface mineralisation of hydroxyapatite in modified simulated body fluid (mSBF) with higher amounts of hydrogencarbonate ions. *Colloids and surfaces A,* 210: p.41-48.

Ebrahimpour, E., M. Johnsson, C. F. Richardson & G.H. Nancollas. (1993). The characterization of HA precipitations. *Journal of Colloind and Interface Science*, 159: p.158-163.

Elliot, J. C. (1994). *Structure and Chemistry of the apatites and other calcium orthophosphates*. 18 vols. Vol. 18, *Studies in Inorganic Chemistry*. Amsterdam: Elsevier.

Gorbunoff, M. J. (1984a). The interaction of proteins with hydroxyapatite. I. Role of protein charge and structure. *Analytical Biochemistry*, 136 (2): p.425-432.

Gorbunoff, M. J. (1984b). The interactions of proteins with hydroxyapatite. II. Role of acidic and basic groups. *Analytical Biochemistry*, 136 (2): p.433-439.

Gorbunoff, M. J. (1984c). The interactions of proteins with hydroxyapatite. III Mechanism. *Analytical Biochemistry*, 136 (2): p.440-445.

Guo, G., Y. Xun, Z. Wang & H. Guo. (2005). Preparation of hydroxyapatite nanoparticles by reverse microemulsion. *Ceramics International*, 31: p.869-872.

Gutman, E. (1997). *Mechanochemistry of Materials*. Cambridge, UK: Cambridge International Science Publishing.

Hattori, T., Y. Iwadate & T. Kato. (1988). *Adv. Ceram. Mater.*, 3: p.426.

Hench, L. L. (1991). Bioceramics: From concept to clinic. *Journal of the American Ceramic Society*, 74 (7): p.1487-1510.

Hench, L. L. (1998). Bioceramics. *Journal of American Ceramic Society*, (7): p.1705-1728.

Hing, K. A., S. M. Best & W. Bonfield. (1999). Characterization of porous hydroxyapatite. *Journal of Materials Science: Materials in Medicine*, (3): p.135-145.

Hirai, T., M. Hodono & I. Komasawa. (2000). The preparation of spherical calcium phosphate fine particles using an emulsion liquid membrane system. *Langmuir,* 16: p.955-960.

Huer, A. H., D. J. Fink, V. J. Laraia, J. L. Arias, P. D. Calvert, A. P. Wheeler, A. Veis & A. I. Caplan. (1992). Innovative materials processing strategies: a biomimetic approach. *Science*, 255:1098-1105.

Ilmori, Y., Y. Kameshima, K. Okada & S. Hayashi. (2005). Comparative study of apatite formation on CaSiO3 ceramics in simulated body flids with different carbonate concentrations. *Journal of Materials Science: Materials in Medicine,* 16: p.73-79.

Ishikawa, K., P. Ducheyne & S. Radin. (1993). Determination of the Ca/P ratio in calcium-deficient hydroxyapatite using X-ray diffraction analysis. *Journal of Material Science: Materials in Medicine*, 4 (2): p.165-168.

Jarcho, M. (1981). Calcium phosphate ceramics as hard tissue prosthetics. *Clinical Orthopaedics and Related Research*, 157: p.259-278.

Jarcho, M., C. H. Bohlen, M. B. Thomas, J. Bobick, J. F. Kay & R. H. Doremus. (1976). Hydroxylapatite synthesis and characterization in dense polycrystalline form. *Journal of Material Science*, 11 (11): p.2027-2035.

Jillavenkatesa, R. A. & S. R. Condrate. (1998). Sol-gel processing of hydroxyapatite. *Journal of Material Science*, 33: p.4111-4119.

Jinawath, S., D. Pongkao & M. Yoshimura. (2002). Hydrothermal synthesis of hydroxyapatite from natural source. *Journal of Materials Science: Materials in Medicine*, 13: p.491-494.

Kawasaki, T., M. Niikura & Y. Kobayashi. (1990a). Fundamental study of hydroxyapatite high-performance liquid chromatography. IIIª. Direct experiemental confirmation of the existence of two types of adsorbing on the hydroxyapatite crystal. *Journal of Chromatography,* 515: p.125-148.

Kawasaki, T., M. Niikura & Y. Kobayashi. (1990b). Fundamental study of hydroxyapatite high-performance liquid chromatography. IIª. Experimental analysis on the basis of the general theory of gradient chromatography. *Journal of Chromatography*, 515: p.91-123.

Klein, C. P. A. T., J. G. C. Wolke, R. C Vriesde & J. M. A. de Blieck-Hogervorst. (1994). Cortical bone ingrowth in grooved implants with clacium phosphate coatings: a gap model study. *Journal of Material Science: Materials in Medicine*, 5 (9-10): p.569-574.

Kokubo, T. (1990). Surface chemistry of bioactive glass ceramics. *Journal of Non-Crystalline Solids,* 120: p.138-151.

Kumta, Prashant N., Charles Sfeir & Dong-Hyun Lee. (2005). Nanostructured calcium phosphates for biomedical applications: novel synthesis and characterization. *Acta Biomaterialia*, 1: p.65-83.

Lacefield, W. R. (1999). Materials characteristics of uncoated/ceramic-coated implant materials. *Advanced Dental Research*, 13: p.21-26.

Landi, E., A. Tampieri, Monica Mattioli-Belmonte, G. Celotti, Monica Sandri, Antonio Gigante, Paola Fava & Graziella Biagini. (2006). Biomimetic Mg- and Mg, CO3- substituted hydroxyapatites: synthesis characterization and in vitro behaviour. *Journal of the European Ceramic Society*, 26: p.2593-2601.

Lavernia, C. & J. M. Shoenuna. (1991). *J. Am. Ceram. Soc.*, 70: p.95.

Layrolle, P., A. Ito & T. Tateishi. (1998). Sol-gel synthesis of amorphous calcium phosphate and sintering into microporous hydroxyapatite bioceramics. *Journal of the American Ceramic Society*, 81 (6): p.1421-1428.

Lim, G. K., J. Wang, S. C. Ng & L. M. Gan. (1996). Processing of fine hydroxyapatite powders via an inverse microemulsion route. *Materials Letters*, 28: p.431-436.

López-Mancipe, A., J. Gómez-Morales & R. Rodríguez-Clemente. (1998). *Adv Mater*, 10 (1): p.49.

Lowenstam, H. A. & S. Weiner. (1989). *On Biomineralization*. Oxford, England: Oxford University Press.

Madsen, H. E. L. & G. Thodvadarson. (1984). Precipitation of calcium phosphate from moderately acid solution. *Journal of Crystall Growth*, 66 (2): p.369-376.

Mateus, A. Y. P., C. C. Barrias, C. Ribeiro, M. P. Ferraz & F. J. Monteiro. (2008). Comparative study of nano-hydroxyapatite microspheres for medical applications."*Journal of Biomediocal Material Research,* 86A: p.483-493.

McCormick, P. G., & F. H. Froes. (1998). The fundamentals of mechanochemical processing. *JOM Journal of the Minerals, Metals and Materials Society*, 50 (11): p.1543-1851.

Muller, Lenka & Frank Muller. (2006). Preparation of SBF with different HCO3- content and its influence on the compositon of biomimetic apatites. *Acta Biomaterialia*, 2: p.181-189.

Murugan, R. & K. Panduranga Rao. (2002). Controlled release of antibiotic from surface modified coralline hydroxyapatite. *Trends in Biomaterials & Artificial Organs,* 16 (1): p.43-45.

Nordstrom, E. G. & H. Karlsson. (1990). *J. Am. Ceram. Soc. Bull.*, 69: p.824.

Noris-Suárez, Karem, Joaquim Lira-Olivares, Ana Marina Ferreira, José Luis Feijoo, Nery Suáres, María C. Hernández & Esteban Barrios. (2007). In vitro deposition of hydroxyapatite on cortical bone collagen stimulated by deformation-induced piezoelectricity. *Biomacromolecules*, 8: p.941-948.

Ogiso, M. (1998). Reassessment of long-term use of dense HA as dental implant: case report. *Journal of Biomedical Materials Research: Applied Biomaterials,* 43 (3): p.318-320.

Ohtsuki, C., S.T. Kokubo & T. Yamamuro. (1992). Mechanism of HA formation on Ca-O-SiO2-P2O5 glasses in simulated body fluid. *Journal of Non-Crystalline Solids*, 143: p.84-92.

Osaka, A., Y. Miura, K. Takeuchi, K. Asada & K. Takahashi. (1991a). Calcium apatite prepared from calcium hydroxyapatite and orthophosphoric acid. *Journal of Material Science: Materials in Medicine*, 2 (1): p.51-55.

Osaka, Y., M. Miura, M. Takeuchi, K. Asada & K. Takahashi. (1991b). *Journal of Material Science: Materials in Medicine*, 2: p.51.

Oyane, Ayako, Kazuo Onuma, A. Ito, Hyun-Min Kim, Tadashi Kokubo & Takashi Nakamura. (2003). Formation and growth of clusters in conventional and new kinds of simulated body fluids. *Journal of Biomedical Material Research: Part A*, 64A: p.339-348.

Panda, R. N., M. F. Hsieh, R. J. Chung & T. S. Chin. (2003). FTIR, XRD, SEM and solid state NRM investigations of carbonate-containing hydroxyapatite nano-particles synthesized by hydroxide-gel technique. *Journal of Physics and Chemistry of Soilds*, 64: p.193-199.

Phillips, M. J., J. A. Darr, Z. B. Luklinska & I. Rehman. (2003). Synthesis and characterization of nano-biomaterials with potential osteological applications. *Journal of Materials Science: Materials in Medicine*, 14: p.875-882.

Piattelli, A. & P. Trisi. (1994). A light and laser scanning microscopy study of bone/hydroxyapatite-coated titanium implants interface: histochemical evidence of unmineralized material in humans. *Journal of Biomedical Materials Research*, 28: p.529-536.

Pileni, M. P. (2003). The role of soft colloidal templates in controlling the size and shape of inorganic nanocrystals. *Nature Materials*, 2 (3): p.145-150.

Ribeiro, C. C., C. C Barrias & M. A. Barbosa. (2006). Preparation and characterisation of calcium-phosphate porous microspheres with a uniform size for biomedical applications. *Journal of Materials Science: Materials in Medicine*, 17: p.455-463.

Rodriguez-Lorenzo, L. M. & M. Vallet-Regi. (2000). Controlled crystallization of calcium phosphate apatites. *Chemistry of Materials*, 12: p.2460-2465.

Rouer, J., C. Viguie, M. H. Heughebaert & J. Heughebaert. (1993). *Journal of Material Science: Materials in Medicine*, 4: p.76.

Ruys, A. J., M. Wei, C. C. Sorrell, M. R. Dickson, A. Brandwood & B. K. Milthorpe. (1995). Sintering effects on the strenght of hydroxyapatite. *Biomaterials*, 16 (5): p.409-415.

Sarda, S., M. Heughebaert & A. Lebugle. (1999). Influence of the type of surfactant on the formation of calcium phosphate in organized molecular systems. *Chemistry of Materials*, 11: p.2722-2727.

Soni, P.R. (2001). *Mechanical Alloying Fundamentals and Applications*. Cambridge, UK: Cambridge International Science Publishing.

Sonoda, K., T. Furuzono, D. Walsh, K. Sato & J. Tanaka. (2002). Influence of emulsion on crystal growth of hydroxyapatite. *Solid State Ionics*, 151: p.321-327.

Spanos, N., V. Deimede & P. G. Koutsoukos. (2002). Functionalization of synthetic polymers for potential use as biomaterials: selective growth of hydroxyapatite on sulphonated polysulphone. *Biomaterials*, 23 (3): p.947-953.

Suchanek, W., H. Suda, M. Yashima, M. Kakihama & M. Yoshimura. (1995). Biocompatible whiskers with controlled morphology and stoichiometry. *Journal of Material Research*, 10 (3): p.521-529.

Sugiyama, Shigeru, Hinori Matsumoto, Hiromu Hayashi & John B. Moffat. (1999). Decomposition of tetrachloromethane on calcium hydroxyapatite under methane oxidation conditions. *Applied Catalysis B: Environmental,* 20: p.57-66.

Suzuki, T., K. Ishikawa & M. Miyake. (1984). Synthetic hydroxyapatite as inorganic cation exchangers. *Journal of Chemical Society, Faraday Trans. 1 ,* 80: p.3157-3165

Tanaka, Yosuke, Yoshihiro Hirata & Ryuichi Yoshinaka. (2003). Synthesis and characteristics of ultrafine hydroxyapatite particles. *Journal of Ceramic Processing Research*, 4 (4): p.197-201.

Tiselius, A., S. Hjertén & O. Levin. (1956). Protein chromatography on calcium phosphate columns. *Archives of Biochemistry and Biophysics*, 65: p.132-155.

Uehara, T., K. Takaoka & K. Ito. (2004). Histological evidence of osseointegration in human retrieve fractured hydroxyapatite-coated screw-type implants: a case report. *Clinical Oral Implants Research*, 15: p.540-545.

Walsh, D. & S. Mann. (1996). Chemical synthesis of microskeletal calcium phosphate in biocontinuous microemulsions. *Chemistry of Materials*, 8: p.1944-1953.

Wang, Feng, Mu-Sen Li, Yu-Peng Lu, Yong-Xin Qi, & Yu-Xian Liu. (2006). Synthesis and microstructure of hydroxyapatite nanofibers synthesized at 37ªC. *Materials Chemistry and Physics*, 95: p.145-149.

Wang, R. Z., F. Z. Cui, H. B. Lu, H. B. Wen, C. L. Ma & H. D. Li. (1995). Synthesis of nanophase hydroxyapatite/collagen composite. *Journal of Materials Science Letters*, 14 (7): p.490-492.

Webster, T. J., R. W. Siegel & R. Bizios. (2000). Enhanced functions of osteoblasts on nanophase ceramics. *Biomaterials*, 21: p.1803-1810.

Wu, Hsi-Chin, Tzu-Wei Wang, Jui-Sheng Sun & Wen-Hsi Wang. (2007). A novel biomagnetic nanoparticle based on hydroxyapatite. *Nanotechnology*, 18: p.165601-165609.

Yan, Li, Yadong Li, Zhao-Xiang Deng, Jin Zhuang & Xiaoming Sun. (2001). Surfactant-assisted hydrothermal synthesis of hydroxyapatite nanorods. *International Journal of Inorganic Materials,* 3: p.633-637.

Yoshimura, M. (1998). Importance of soft solution processing for advanced inorganic materials. *Journal of Materials Research*, 13 (4): p.796-802.

Yuan, Z. Y., J. Q. Liu & L. M. Peng. (2002). Morphosynthesis of vesicular mesostructured calcium phosphate under electron irradiation. *Langmuir*, 18: p.2450-2452.

Zahouily, M., Y. Abrouki, B. Bahlaouan, A. Rayadh & S. Sebti. (2003). Hydroxyapatite: new efficient catalyst for the Michael addition. *Catalysis Communications*, 4: p.521-524.

Zhu, Xialong, Oliver Eibl, Chirstoph Berthold, Lutz Scheideler & Jürgen Geis-Gerstorfer. (2006). Structural characterization of nanocrystalline hydroxyapatite adhesion of pre-osteoblast cells. *Nanotechnology*, 17: p.2711-2721.

Zong, S. & K. E. Gonsalves. (1997). *Journal of Material Science: Materials in Medicine*, 8: p.25.