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Synthesis and Extraction of Hydroxyapatite Grafts from Animal Sources

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Introduction

Bone is the second mainly implanted tissue after blood in the human body system. The biomechanical properties of bone result from its complex structural arrangement of organic (20-30%) (Collagen, Noncollagenous Proteins and Lipids), inorganic components (60-70%) and 5% water [1]. Bone inorganic matrix is mainly composed of hydroxyapatite (HA; Ca10 (PO4)6(OH)2) with 3% to 8% of carbonate content [2]. Mechanical properties of the bone are characterized by Hydroxyapatite (HA) compositions, its crystalline structure, morphology, particle size and orientation. Graft materials used for formation of bone tissue include autologous bone, allogenic bone, xenograft tissue and artificial bone. Although hydroxyapatite has been used for many years as a non- absorbable biocompatible bone substitute, more recently the focus has been on the development of absorbable bone substitutes. When the area of a bone defect is filled with hydroxyapatite, the cycle of bone resorption by osteoclasts and bone formation by osteoblasts resumes at the bone surface, leading to the formation of new autologous bone. The highly porous and nanometer -sized granules increase the activity of osteoblasts, resulting in more rapid proliferation, adhesion and differentiation of bone cells. However, it has been reported that the high porosity and small size of these hydroxyapatite granules interferes with resorption of the graft and ingrowth of bone.

Mechanical properties of the bone are characterized by HAp compositions, its crystalline structure, morphology, particle size and orientation. The crystals are nanometer-sized needle like or rod-like shapes (average length - 50 nm, width - 25 nm and thicknesses 2-5 nm) scattered in the organic matrix and composed of OH− , Ca2+ and PO4 3− groups (closely packed in hexagonal arrangement; space group - P63/m). Special behavior and bioactivity of biological appetites could vary with HAp dimensions, low crystallinity and presence of carbonate ions in the lattice. Hence, hydroxyapatite has fascinated interest of several researchers from past few decades [3, 4].

Chemical Properties of Hydroxyapatite

HA is used as a bone substitute because of its chemical similarities with the natural bone. The major composition of bone is a mineral phase (69 wt %), an organic matrix (22 wt %), and water (9 wt %). Bone is the major calcified tissue present in mammals and is a ceramic-organic bio nanocomposite that has a complex structure. HA with a general formula of Ca10 (OH)2(PO4)6 is much similar to an inorganic component of bone matrix. Because of this close similarity, extensive research is ongoing to use HA as bone regenerative material with mCa/P ratio of 1.67. The pure HA powder is white, whereas naturally occurring HA can also have brown, yellow, or green colorations, comparable to the discolorations of dental fluorosis. In biological systems, HA occurs as the principal inorganic constituent of normal (bone, teeth, fish enameloid, and some species of shells) and pathological (dental and urinary calculus and stones) calcifications [2].

Physical Properties

The mechanical properties of HA depend on porosity, density, sinter ability, crystal size, phase composition, and so on. The bending, compressive, and tensile strength values of HA ceramics lie in the range of 38-250, 120-150, and 38-300 MPa, respectively [5]. Young's modulus of dense HA ceramics varies from 35 to 120 GPa, depending on the residual porosity and impurities. Weibull's modulus of dense HA ceramics lies in the range 5-18, characteristic of brittle materials. The Vicker's hardness of dense HA ceramics is 3-7 GPa. The mechanical properties of HA bioceramics strongly depend on the microstructure and sintering ability; densely sintered bodies with fine grains are tougher and stronger than porous ones with larger grains [6].

Biological Properties

HA bioceramics have been widely used as artificial bone

substitutes because of their favorable biological properties, which include biocompatibility, bioaffinity, bioactivity, osteoconduction, osteointegration and osteoinduction (in certain conditions). Hydroxyapatite contains only calcium and phosphate ions and therefore no adverse local or systemic toxicity has been reported in any study. When implanted, newly formed bone binds directly to Hydroxyapatite through a carbonated calcium deficient apatite layer at the bone-implant interface [7].

Hydroxyapatite surface supports osteoblastic cell adhesion, growth, and differentiation, and new bone is deposited by the creeping substitution from the adjacent living bone. Hydroxyapatite scaffolds can also serve as delivery vehicles for cytokines with a capacity to bind and concentrate bone morphogenetic proteins .The interaction of apatite with biological tissues is an important aspect for regeneration. The concepts in the mineralization and tissue interaction are changing because of the change in production technology, size, nature of material, and so on. The beginning of bone regeneration has started with scaffold. The term biomimetic was coined by Otto Herbert Schmitt in 1950s. The tissue interactions of Hydroxyapatite are important. It is necessary to understand its *in vivo* host responses. In general, the mechanism of action of a biomaterial is considered to be biocompatible, bioinert, biotolerant, and bioactive, and includes bioresorbable materials. These shifts in understanding have occurred due to the changes in the properties and production technology and the deeper understanding of material interaction with the tissues. The advanced front of nanotechnology results in cutting edge production of Hydroxyapatite in a much bioactive or bioresorbable manner. The tissue reaction to any foreign body, even though it is biocompatible, will form a capsule thus it will be isolated. Bioinert materials will not show any positive interaction nor release any toxic constituents. The body or host tissue will separate such materials through encapsulation, which measures the bioinertness of material. A bioactive material will dissolve slightly, but it forms a biological apatite before it interacts with tissues at the atomic level, which results in the formation of chemical bonds directly with bone. This phenomenon provides good stabilization for the materials that are subjected to mechanical loading. Bioresorbable material dissolves over a period of time so that new tissues will grow into surface irregularities. The bioresorbable materials are used as scaffolds that allow substitution and act as filling material. These reactions depend on the nature of the material, such as porosity. Recently, concepts are introduced where bioactive material are made into bioresorbable and bioresorbable into bioactive. The use of HA with Ca/P ratio of 1.0-1.7 is nontoxic and neither has it induced any foreign body reaction. The nature of healing mimics fracture healing. Hydroxyapatite has displayed an ability to directly bond with bone. Sometimes, the micromovement of implants may lead to inflammation because of disruption of large microvessels that have grown into the pores of the implant. HA also exhibits the property of osteoinductivity.The mechanism of bone induction by a synthetic material is still not clear, but various factors such as microporosity, surface area, geometry, and topography are important,of which microporosity has a positive effect on increasing ectopic bone formation. Some studies have shown that osteoinduction is brought about by the concentration of bone growth factors from circulating biological fluids [8].

Geometry of Hydroxyapatite is also a critical parameter in bone induction. Nano structured Hydroxyapatite, rough surfaces are found to cause asymmetrical division of stem cells into osteoblasts, which is important for osteoinduction. Biodegradation of Hydroxyapatite is usually initiated by changes in the surrounding biofluids and adsorption of biomolecules. The physicochemical dissolution process depends on the surface area to volume ratio, fluid convection, acidity, and temperature. The dissolution is usually inversely proportional to the Ca/P ratio, purity, crystal size, and surface area. Usually, Hydroxyapatite is more stable than other calcium orthophosphates such as Tricalcium phosphates. Bioresorption is usually mediated by osteoclast cells, sometimes by macrophages. The biodegradation kinetics depend on the Hydroxyapatite /Tricalcium phosphate ratio. The higher the ratio, the lower the degradation. The incorporation of ions either increases or decreases the solubility of Hydroxyapatite and CDHA (Calcium deficient hydroxyapatite). Bioactive materials form a chemical bond. The roughness and biomaterial porosity are considered important factors for bonding. During the interfacial reactions of bioactive materials, the proteins will be adsorbed on the HA surface. The extent and interconnectivity of pores influence bone ingrowth and blood vessel formation. A minimal pore size of approximately 50 μm has been estimated for blood vessel formation and approximately 200 μm for osteoid growth. The pore dimension, approximately 100 μm and 50 μm, also showed bone in growth. The pore size and effects are as follows: 1 μm is responsible for bioactivity, interaction with proteins, and attraction of cells; 1-20 μm leads to the orientation and directionality of cellular and bone in growth; 100-1,000 μm helps in mechanical strengthening and functionality; 1,000 μm influences the shape and esthetics of the implant. The degree of porosity regulates the bioactivity of graft substitutes, which controls the rate of bone regeneration, local environment, and equilibrium of new bone at the repair site. The pore interconnectivity, geometry, topography, and porosity modulate osteogenesis, which synergistically promotes the osteoconductivity or the inductivity potential of bone graft. The excellent biocompatibility, possible osteoinductivity and high affinity for drugs, protein, and cells make these tissue-engineering applications very much functional [8].

Osteoinductive Properties

Osteoconduction and osteoinduction properties of HA scaffolds are well known. Osteoinduction occurs because of the stimulation of the host mesenchymal stem cells. These stem cells then differentiate into bone-forming osteoblasts. Extensive studies have been conducted over the past several years to understand the osteoinduction potential of HA. Osteoinduction has been observed in several independent studies in various hosts such as dogs, goats, and baboons. Ripamonti et al have conducted extensive work on the long-term use of HA implants in the nonhuman primate Papio ursinus. Their studies indicate spontaneous bone formation in non-osseous sites. In one study, they used coral-derived calcium carbonate that was converted to HA by a hydrothermal reaction. Constructs of HA and calcium carbonate (5% and 13% HA) exhibiting different morphologies (rods and disks) were implanted into the heterotopic rectus abdominus or into orthotopic calvarial defects, respectively. Different time points were assessed during this one-year study and, in all instances, induction of bone in the concavities of the matrices was detected. After a year, resorption of the calcium carbonate/HA as well as deposits of newly formed bone was visible. Ripamonti also reported the use of HA-coated Ti implant in an 8-month *in vivo* trial in P. ursinus, where osteoinduction was also observed. The rationale in using HA coatings as a means of fixation for orthopedic and dental implants has been known as early as the 1980s. HA as a surface coating attempts to improve bone fixation to the implant and thus increases the lifetime of metallic implants. Higher osteoblast activity and in vitro increased collagen levels seen in cells growing on HA-coated Ti, *in vivo* HA coat resulted in higher

bone-implant contact area. Enhancing the ingrowth of mineralized tissue improved the biological fixation, biocompatibility, and bioactivity of dental implants. The deposition can be achieved through plasma spraying, sputter coating, pulsed laser deposition, dynamic mixing method, dip coating, sol-gel, electrophoretic deposition, electrochemical deposition, and biomimetic coating with various advantages and disadvantages. Thinner HA layers, in the nanometer range, revealed increased cellular response than thicker HA layers. Recently, biomimetic HA-polymer composite scaffolds have been widely explored for bone regeneration [9, 10].

Different Sources of Natural HA

Natural hydroxyapatite is usually extracted from biological sources or wastes such as mammalian bone (e.g. bovine, camel, and horse), marine or aquatic sources (e.g. fish bone and fish scale), shell sources (e.g. cockle, clam, eggshell, and seashell), and plants and algae and from mineral sources (e.g. limestone). Stoichiometric HA is basically composed of calcium and phosphorus with molar ratio of Ca/P equal to 1.67. This ratio has been proven to be the most effective in promoting bone regeneration. Natural HA is non-stoichiometric and is either deficient in calcium or phosphorus. Calcium positions are the most common vacancy in HA where cations such as Na, Mg, and Al are substituted in the calcium positions, while carbonate ions can substitute for either phosphate or hydroxyl ions while fluoride ions substitute for hydroxyl ions. The presence of trace elements in some natural HA mimics the apatite produced from human bone. The trace elements are essential in the regeneration of the bone and accelerates the process of bone formation. Research by Balamurugan et al. proved that 3-5 mol% silicon added to synthetic hydroxyapatite (Si-HA) increased cell growth density, which enhanced osteoblast growth [11].

Research by Capuccini et al. showed that the presence of 1-10% of strontium ions in synthetic HAp enhanced osteoblast activity and differentiation and also inhibited osteoclast proliferation and production [12]. Carbonate-substituted hydroxyapatite was also proven to enhance osteogenesis by enhancing bioresorption [13].

The use of HA extracted from natural sources can be considered to be an environmentally friendly, sustainable, and economical process to fabricate these materials since these materials are available in large quantities. This can result in positive contributions to the economy, environment, and to general health [14].

Mammalian Sources

Among mammalian sources, the extraction of HA from bovine bone was frequently reported in literature compared to other sources such as camel, horse and, porcine. The cortical part of the femoral bone is usually used because they are morphologically and structurally similar to human bone. The properties of the extracted HA, such as the Ca/P ratio, size, shapes and crystalline phases of Ca-P differ with the applied extraction methods and parameters such as calcination temperatures and pH. Generally, most literature have reported that pretreatment of the bone is usually done before proceeding with the extraction method. The pretreatment involves washing and removing the dirt, fats, protein, and other components such as bone marrows and soft tissues. Some literature reported the usage of boiling water to remove organic components from the bone by boiling for 8 h or more

A combination of boiling and washing with solvents such as acetone and chloroform have been employed for the pretreatment of bone .Another pretreatment method that has been widely used is washing the bone alternatively with surfactant and alkali solutions to remove the soft tissues and decellularise it [15].

The bone is also cut into smaller pieces before or after removing the organic constituents. Most of the methods for extraction of HA from mammalian bones used the calcination method, which is either the sole process or a combination of calcination with other methods. The calcination process involves heating the bone in a furnace at different temperatures of up to 1400° C in order to completely remove the organic matter and kill the pathogens, which may be present. Barakat et al. employed the alkaline hydrothermal hydrolysis treatment to extract HA from bovine bone 16. The extracted HA was heated to 250° C for 5 h resulting in the formation of nano flake HA with Ca/P ratio of 1.86. Meanwhile extracted HA using thermal decomposition method where samples were heated at two different temperatures (750° C and 850° C) for 6 h leading to the formation of HA with two-particle size. The Ca/P ratio was higher than 1.67 owing to the exchange of ions such as carbonate in the HA structure [16].

XRD patterns showed that HAp and β-TCP were formed during the calcination. Previous research showed that HA decomposes to β-TCP at temperatures >1000 C, but research by Sun et al. showed that this process can occur at 850 °C and 750 °C leading to formation of β-TCP. Thus, Sun et al. concluded that the presence of β-TCP was not formed from the decomposition of HA and thus may have originated from the bovine bone. Sun et al. used alkaline heat treatment to extract HA from bovine bone where the sample was treated with 20% sodium hydroxide and heated at 80° C for 10 h followed by washing and freeze-drying. This process resulted in low crystalline irregularly shaped HA particles of 20-100 μm. This process resulted in the presence of minor element such as Mg and Na and trace elements including Zn, Sr, and Ba in the sample [17].

Elemental analysis showed that besides calcium and phosphorus, trace elements such as Na, Mg, Sr, Fe, Al, and Zn were present in the calcined camel and horse bone. The presence of trace elements in the HA can enhance and accelerate the growth of the bone. Prior research has shown that minor and trace elements can enhance the formation of new bone during in vitro and *in vivo* test [18]. Ruksudjarit et al. used a combination of calcination with vibro-milling technique to extract the HAp from bovine bone. The bone then was calcined at 800◦ C for 3 h before multiple milling steps were used. Two steps of milling were used, namely ball milling (24 h) and vibro-milling for different times (0, 1, 2, 4, and 8 h). Ruksudjarit et al stated that the substitution of sodium for calcium in HA enhanced the apatite-forming capacity in Simulates Body Fluid and exhibited excellent osteoconductivity compared to pure HA [19].

Research by Rahavi et al. on the calcination of camel and horse bone at 700 °C for 2 h resulted in the production of irregularly shaped nano-sized HA. The size of the extracted HA from the camel and horse bone were 97 nm and 28 nm respectively. The results showed that the calcination produced crystalline HA with Ca/P ratio of 2.036 for camel and 2.131 for horse, both of which are higher than that seen for stoichiometric HA [20].

Ofudje et al. used calcination to extract HAp from pig bone where clean and dried pig bone were calcined at 600° C, 800 °C, and 1000° C prior to pre-treatment. The results showed that pure crystalline HA was produced with a rod-like morphology (38-52 nm length and 7-13 nm width). The Ca/P ratio of the sample after calcining at 1000 °C was 1.88. Most biological apatite is non-stoichiometric owing to the presence of the trace elements that replace the Ca in the apatite lattice [21].

Calcination method was the most popular method for fabrication compared to others. The calcination process removes the organic constituent in the bone by the thermal process. The organic matter is converted to carbon dioxide and ash (calcium phosphate compounds). This calcium phosphate usually does not decompose at temperature below 1200° C and will be present as different calcium phosphate (CaP) phases. Increasing temperatures increases the crystallinity of the HA particles along with other calcium phosphate phases such as β-TCP. On the other side, use of high calcination temperatures will remove all pathogens and prevent the possibility of transmission of diseases such as bovine spongiform encephalopathy and Creutzfeldt Jakob disease. Researches have concluded that pathogens cannot survive at temperature above 800° C. Other methods such as alkaline heat treatment have been used to extract HAp from mammalian bone. In this method, the alkaline solution usually NaOH is used to remove the organic matter from the bone. The NaOH solution hydrolyzes the organic component in the bone and the remaining calcium phosphate is rinsed and separated using filtration. However, alkaline heat treatment produces low crystallinity HA compared to calcination. Sun et al. revealed that the crystallinity of HA produced using alkaline heat treatment was much lower than that seen for calcined HA. The use of high calcination temperatures can result in β-TCP phase formation from HA which can reduce the Ca/P ratio. The presence of CaO can increase the Ca/P ratio. The aim of achieving Ca/P values close to stoichiometry since either the trace elements or β-TCP can enhance the properties of these materials for bone implant purposes. The morphological analyses of the HA extracted from the mammalian bone show that the particles are mostly irregular in shape, with some studies showing the presence of rods, flakes, needles, and plate-like shapes. The shape variation is believed to not be affected by the method or source. For example, calcination of same source of bone could produce the various shapes of HA such as rod-like, spherical, and needle like. In addition, the rod shape HA could be produced using different extraction methods such as alkaline hydrolysis and combination method. It can be concluded that, there is no relation between the morphology of HA with the extraction method and source. The size of HA obtained did not show any correlation with the extraction method. The use of additional milling helped to reduce the size of the HA particles to the nanometric size which is close to that of human HA. In addition, the nanosized particles have advantages in terms of high surface activity and ultra fine structures, higher bioactivity, and better resorbability than micron-sized particles.

Hydroxyapatite from Fish origin

In the last years, HA from fish bone and scales has emerged as an alternative to substitute synthetic and bovine HA, because similar chemical properties have been achieved by simple and inexpensive methods. It has been demonstrated that fish sources are safe and present low risks of disease transmission. Additionally, fishes are abundant in the environment, and the application of their byproducts is suitable for biomedical application since it would reduce environmental pollution and threats of biohazards to humans. Many different fish species have been used to obtain HA, such as salmon, carp, Japanese anchovy, sardine, tilefish, tuna, among others. For this purpose, many different protocols of extraction and methods for chemical analysis have been proposed in the literature, of which its goal is to obtain and to characterize HA extracted from fish. The majority of the protocols are based on the calcination method. In 1995, Hamada et al have investigated the physico-chemical properties of HA extracted from 15 different species of fishes using the X-ray diffraction (XRD) and elemental analysis. It seems that, the calcination method is a simple, cheap, and effective method to extract HA. Bone samples were boiled in water, cleansed, dried at room temperature, and through an electric furnace, ashed at 600o C, for 3 h. After cooling, bone ashes were grounded in a mortar to a powder, and submitted to physico-chemical analysis. XRD analysis demonstrated characteristic peaks of HA in the samples from sea bream, horse mackerel, carp, shark, cattle, swine, and fowl, while the peak of tricalcium phosphate (TCP) was confirmed only in Japanese anchovy. Both peaks of HA and TCP were verified in sardine, mackerel, tilefish, croaker, triggerfish, lizard fish, Spanish mackerel, flying fish, congereel, and flat fish. Moreover, the analysis of elemental contents demonstrated that the most abundant element in the fish bones was calcium followed by phosphorus. Sodium and magnesium were present in some of the species as well [22].

Venkatesan and Kim evaluated different temperatures to calcinate fish bones (200°C to 1200 °C) for obtaining HA. Similar procedures were performed to clean bone samples, and 200-μm particle size of bone samples were placed in mold and heated in a furnace, at temperatures ranging from 200 °C to 1200°C. XRD analysis demonstrated that fish bone heated at 100 °C shows an amorphous nature, with low crystallinity of the HA. Under heat treatment at 400-900 °C, bone was initially transformed to a well crystallized HA. The presence of TCP phases in the sample sintered at 1200 °C indicated the decomposition of HA. Thus, it is possible to develop pure phase HA at temperature below 1200 °C and a sintered composite of TCP/HA with heating at 1200°C [23].

They used both thermal calcination method and the alkaline hydrolysis method, and then they compared the products by means of chemical, physical, and microscopic analysis. For alkaline hydrolysis method, grounded tuna bone was treated with sodium hydroxide, at 250 oC for 5 h for proper removal of organic moieties. The mixture was then filtered in a suction pump with continuous washing with water until the pH was neutral. The product was dried in an oven at 100 oC. For thermal calcination method, tuna bone was placed in a silica crucible and subjected to a temperature at 900 oC in an electrical muffle furnace in the presence of air atmosphere for 5 h. Charactherization was performed by thermal gravimetric analysis (TGA), Fourier transform infrared spectroscopy (FT-IR), XRD, microscopy, and transmission electron microscopy (TEM). FT-IR and TGA analysis showed that collagen and organic moieties have been eliminated by the proposed methods. XRD analysis demonstrated that thermally calcinated bone was more crystalline when compared to the alkaline hydrolyzed bone. TEM demonstrated that HA obtained from thermal calcination method was crystalline, with dimensions of 0.3-1.0 μm, whereas HA obtained through the alkaline hydrolysis method was structured in nanocrystals, with 17-71 nm length and 5-10 nm width, respectively. As a conclusion, authors stated that alkaline hydrolysis is the best approach for nanostructured arrangement rather than the thermal calcination method. Overall, alkaline hydrolysis method takes an advantage of deriving HA with required physicochemical properties [23].

Fish scales have also been used as a source for HA extraction. Mondal et al collected fish scales from fresh water fish (Labeo Rohita), and submitted them to a process to deproteinize the material through external washing with HCl for 24 h. Thus, fish scales were washed several times, and treated with NaOH solution. The remaining material was dried at 60°C and calcined to different temperatures for producing HA ceramics. XRD patterns of synthesized HA demonstrated sharp peaks of powders at high calcination temperature confirming the complete crystallization

of the powder [24].

Hydroxyapatite from Eggshells

An eggshell is composed of three layers, which are the cuticle, spongia, and lamella. These contain protein fibers and calcium carbonate. The weight of the eggshell accounts for less than a third of that of the egg, and it is composed mainly of calcium compounds (over 90%) and small traces of organic matter. The eggshell is a great source of calcium or dietary supplement. Eggshell consists of calcium carbonate (94%), calcium phosphate (1%), organic matter (4%) and magnesium carbonate (1%). Eggshell derived hydroxyapatites have been reported to be biocompatible and efficient bone graft substitute, which is produced in a very economical way.

Lee et al, (2003) did a study on fabrication of calcium phosphate biocermaics like hydroxyapatite and β-tricalcium phosphate using recycled eggshell and phosphoric acid by new wet chemical method and observed the mixing ratios of calcium phosphate and phosphoric acid and calcination temperature. They stated that hydroxyapatite and β-tricalcium phosphate were stably synthesized in the mixing ratios of 1: 1.1-1:1.2 and 1:1.3-1:1.5 respectively [25].

Dupoirieux et al, (1995) did a pilot study on a new bone formation in rabbits and rats using powdered eggshell in maxillofacial surgery and stated that eggshell powder exhibited excellent biocompatibility, no risk of viral transmission while it is a complication with allografts, no toxicity, inexpensive and can be found in unlimited quantity [26].

Hydroxyapatite from Marine Shells

Crab shells

Crabs shells containing calcium carbonate ($CaCO_3$) are very abundant; amount 40-70%, varies according to the species. Calcium carbonate can be further processed into calcium hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$].

The crystals structure of hydroxyapatite will be better by using CaO as a precursor of calcium. However, the use of these compounds also produces carbonate apatite [$Ca_{10}(PO_4)_6CO_3$] in a fairly large percentage. This is because the calcination process cannot eliminate carbon dioxide (CO_2) in $CaCO_3$ so that there can be reaction with the precursor phosphate. Crabs shells Portunus pelagicus waste was cleaned with distilled water and dried at room temperature. Furthermore, to transform crabs shells into $CaCO_3$ and then into CaO, calcinations were performed on the samples at 1000° C temperature for 5 hours at a rate of temperature rise 5□ C/minute. Calcium oxide (CaO) obtaining dominated as result of calcinations and then made suspensions in 100 ml of distilled water with a calcium concentration of 0.3 M. The suspensions reacted by drop wise with a 100 mL 0.2 M of $(NH_4)_2HPO_4$ solution through the coprecipitation method, at temperatures around 40□ C while the solution was stirred for 2-5 hours. The precipitation was allowed to stand overnight or 24 hours at room temperature, and the precipitate is filtered with a Whatman filter paper number 40 and dried at 110□ C for 5 hours. The pure hydroxyapatite obtained by sintering to the dried precipitate at various temperatures of 500°-900° C for 4 hours . The material was washed with distilled water and then dried at a temperature of 110□ C. The characterization of the compounds was performed by using X-ray diffraction (XRD), FTIR, and SEM-EDXA [27].

Hydroxyapatite from Seashells

A seashell can be defined as the hard outer layer from the body of an animal that lives in the sea such as marine mollusks; mussels, oysters, scallops, clams, snails and cockles [28]. Mussel shells are one of the most abundant by-products produced by fish industries. Despite their potential as valuable compounds source, they are not sufficiently valorized—indeed, in most cases, they are simply disposed off. As with other seashells, mussel shells can also be employed to prepare HA-based materials; however, very few studies investigating this preparation are reported in the literature.

Variations in the shell architecture are observed in the mussels both within a species and among different species. The unique morphological features of all mussels (Mollusca: Bivalvia) is the presence of two symmetrical calcareous valves, constituting the shells, connected by a calcified leathery hinge. The toughest calcareous shells of the mussels vary extensively in shape, size, colour and biomass. While calcium carbonate is the dominant chemical constituent of the shells, minor inorganic trace elements are common in different species of bivalve. The majority of seashell types studied reported calcium oxide (CaO) contents rather than $CaCO_3$ contents. This suggests the removal of organic material from seashells may be difficult and heating to elevated temperatures results in a purified form. For instance, heated oyster shells have been reported to contain CaO (51.06%), with fractions of SiO_2 (2%), Al_2O_3 (0.50%), Fe_2O_3 (0.20%), MgO (0.51%), SO_3 (0.60%), K_2O (0.06%), Na_2O (0.58%), TiO_2 (0.02%), Mn_2O_3 (0.02%), P_2O_5 (0.18%) and SrO (0.09%) as a well as a 44.16% loss of CO_2 and organic materials [29].

Hydroxyapatite from Corals

The use of coral as a bone graft substitute dates back to early 1970s. Initially they were used in stabilising of non- cemented prostheses. Hydroxy apatite can be manufactured from natural reef building coral skeleton by a hydrothermal exchange reaction, where the trabecular, bone imitating structure of the coral remains unchanged and the calcium carbonate (CC) skeleton is converted to calcium phosphate, the main inorganic salt of bone.

The original coral skeleton, consisting of, can also serve as bone substitute. The main difference between these structurally identical materials is that biodegradation takes place much more slowly with HA than with. Biodegradation should not occur before the implant has filled with bone. Very short resorption times, even a few weeks, have been reported for Calcium carbonate. This accentuates the importance of fast bone ingrowth into the implant. However, both HA and CC lack the capacity to induce bone growth. Adding bone marrow to porous calcium ceramics induces bone formation, even in extraosseal sites.

Each species build a structurally and geometrically typical calcium carbonate skeleton. Choice of the appropriate species therefore enables a desired and constant implant structure to be achieved. Coral reefs are formed by colonies of polyps ranging in size from one millimeter to several centimeters, depending on the species. Coral polyps live in symbiosis with unicellular algae, which photosynthesize compounds essential to the polyps. The outer layer of the polyp is capable of secreting a substance that calcifies in the seawater milieu and serves as matrix for the coral skeleton. The coral polyp lives only in the upper part of the skeleton, moving slowly upwards, leaving an empty skeleton behind. More than 2000 coral species have been described from the intertropical area and, of these, fourteen Scleractinian corals have been studied as possible bone substitutes. The following genera have already

been used as bone grafts: Pocillopora, Acropora, Montipora, Porites, Goniopora, Fungia, Polyphyllia, Favites, Acanthastrea, Lobophyllia and Turbinaria (Bouchon et al. 1995) [30].

Coral resorption is most active in the bone implant contact areas and proceeds centripetally (Braye et al. 1996). Carbonic anhydrase, an enzyme abundant in osteoclasts, plays a key role in the resorption process. Locally it lowers the pH at the osteoclast-implant interface, dissolving the CC matrix (Chétail and Fournié 1969; Gay and Mueller 1974; Guillemin et al. 1981. Resorption can be halted by the administration of the diuretic acetazolamide, a known inhibitor of carbonic anhydrase. Moreover, according to Fricain and coworkers, data suggest that both fibroblasts and macrophages dissolve the coral, and that one of the mechanisms is the intracellular degradation in phagolysosomes (Fricain et al. 1998, a). A prerequisite for the process is direct contact between these cells and the coral matrix (Fricain et al. 1998, b) [31].

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