RESEARCH

Chitosan/hydroxyapatite-based composites

✍ Carlos Peniche[1], Yaimara Solís[1], Natalia Davidenko[1,2], Raúl García[3]

[1]Centro de Biomateriales, Universidad de la Habana, UH
Ave. Universidad e/ G y Ronda, AP 6120, CP 10 600, Ciudad de La Habana, Cuba
[2]Department of Materials Science and Metallurgy, University of Cambridge, Cambridge CB2 3QZ, UK
[3]Instituto de Cerámica y Vidrio, Consejo Superior de Investigaciones Científicas, CSIC
Madrid 28049, España
E-mail: peniche@reduniv.edu.cu

ABSTRACT

In the past 20 years, there has been a growing trend towards the development and use of biomaterials for the repairing and restoration of damaged bone tissue. The calcium phosphate bioceramics have attracted great attention in the field of orthopaedics because of its similarity to the mineral component of bone tissue. They have been used as granules for example in non-load bearing small implants, such as middle ear implants, in coatings on metals as dental implants, as well as in porous implants to stimulate bone growth within the implant; and cements, which are implanted in a paste like form and harden *in vivo*. The clinical disadvantages associated with them are primarily focused on poor mechanical strength and slow resorption kinetics as compared with the surrounding tissue. Therefore, current studies are aimed at creating new formulations combining calcium phosphate compounds with biopolymers, in order to avoid the frequent migration of bioceramic particles from the implant site, reducing potential damage to soft tissue in the vicinity of the implant and to improve biodegradability, curing properties, mechanical strength and injectability. The aims of this paper is to bring a review of the literature concerning chitosan-hydroxyapatite composites for bone restoration, and to mention the main methods of preparation, physico-chemical and biological properties, and tissue engineering techniques using these materials.

Keywords: composite, hydroxyapatite, chitosan, bone tissue, bone tissue engineering

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RESUMEN

Materiales compuestos de quitosana e hidroxiapatita. En los últimos 20 años se observa una tendencia creciente del desarrollo y empleo de biomateriales para la reparación y regeneración del tejido óseo dañado. Las biocerámicas de fosfatos de calcio han despertado gran interés en el campo de la ortopedia debido a su similitud con el componente mineral del tejido óseo. Estas se han utilizado como gránulos en implantes pequeños que no tengan que soportar cargas, como el oído medio; en recubrimientos sobre metales que las refuercen, como los implantes dentales, los implantes porosos para estimular el crecimiento de un hueso dentro del implante, y los cementos que se implantan en estado pastoso y fraguan *in vivo*. Los inconvenientes clínicos asociados con las biocerámicas se centran fundamentalmente en la pobre resistencia mecánica y la lenta cinética de reabsorción en comparación con el tejido circundante. Es por ello que los estudios actuales están encaminados a crear nuevas formulaciones compuestas por fosfatos de calcio y biopolímeros, con vistas a evitar la migración frecuente de las partículas biocerámicas del sitio del implante, disminuir la posibilidad de daños a los tejidos blandos próximos al implante, mejorar la biodegradabilidad, las propiedades de fraguado, la resistencia mecánica y la inyectabilidad de los biomateriales. Los objetivo/s de este artículo son ofrecer una revisión de la información actualizada sobre materiales compuestos (composites) de hidroxiapatita y quitosana como sistemas soporte del tejido óseo; mencionar los principales métodos de preparación, las propiedades físico-químicas y biológicas, y las técnicas de ingeniería de tejidos que utilizan estos materiales.

Palabras clave: composite, hidroxiapatita, quitosana, tejido óseo, ingeniería de tejido óseo

*I*ntroduction

Nowadays, bone infections are among the main problems to be solved in regenerative medicine, due to the increase in people longevity and the high incidence of accidental traumas. According to the World Health Organization (WHO), osteoporosis is the second sanitary assistance health problem worldwide, after the cardiovascular diseases. For instance, up to 60 000 hip fractures are annually reported in Spain, accounting for 20 to 22% of the hospitalization capacity at Orthopedic and Traumatology Services [1].

There is a high prevalence of osteoporosis in Cuba, with a relatively early average onset (before 65 years). More than three quarters of the cases suffer from moderate to severe osteoporosis with a 19% frequency of fractures. In fact, mortality rates are rapidly increasing among the elderly population due to combined incidence of a hip fracture-associated falling and osteoporosis [2]. For example, only in Matanzas province 609 deaths of hip fracture from 2001 to 2007 have been reported. Mortality increased from 1.6 to 2.0% during that period, and the hip fracture/accident death ratio subsequently rose from 32.2 to 46.1. Moreover, the mortality rate of hip fracture proportionally correlated with age increase and gender; the amount of women affected almost doubled the number of men (16.4 vs 9.5)[3]. Currently, osteoporosis has been called the "silent epidemics of the XXI century", because of being the main cause of bone fracture among

✍ Corresponding author

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post-menopausal women and in elderly people [4]. Its affections are not fatal but can seriously deteriorate the patient's quality of life by provoking clinical manifestations of chronic pain, physical disability and esthetic problems.

An alternative to these troubles consists on using bone substitutes, either to replace or to favor the regeneration of the affected tissue. In such cases, bone tissue extracted from the same patient is the most common alternative favoring the best recovery. However, there is a finite amount of bone available to be self-transplanted, also requiring a second surgical intervention. In spite of its limitations, materials used as bone substitutes have included autogenous bone (demineralized bone matrix), metals (titanium, stainless steel and alloys), polymers (methyl polyacrylate), polyesthers (e.g., polylactic and polyglycolic acids), ceramics (alum, calcium phosphates such as hydroxyapatite (HAp), tricalcium and octocalcium phosphate, and silica-based as bioglass and silica-doped nanoapatite), tricalcium β-phosphate (β-TCP) macroporous implants and composites of natural or synthetic HAp with polymers, sometimes combined with biological substances for a more appropriate presentation [5]. Research is increasingly focusing on the design and development of new materials able to stimulate regeneration and repair of damaged bone tissues, aiding a fast recovery of the patient and lowering the high costs of surgery and materials available in the international market.

In Cuba, a natural origin HAp was developed (Coralina, HAP-200), which has been applied successfully as bone graft substitute and for integrated ocular implants [6]. There is also a synthetic HAp (Apafil-G), being used in bone defect implants with good results [5]. Other HAp- and porogenic additives-based calcium phosphate cements (CPCs) were also developed.

Nevertheless, as referred in the specialized literature, there has been a significant trend during the last years on the development of ceramics- and polymer-based scaffolds for tissue engineering. This is due to the excellent osteoinductive properties of ceramic materials, instead of its low degradation, low mechanical resistance and hard to be mold to fit the physical and geometric requirements of the given site or bone defect. At the same time, biopolymers bear low osteoinductivity but with better mechanical and degradation properties. Thus, biopolymer-based calcium phosphate composites allow incorporating advantageous properties of both types of components [7].

Chitosan (CS) is one of the components most frequently used to prepare calcium phosphate composites, because of its biocompatibility, biodegradation and innocuousness. Due to the interest on composites based on this biopolymer, here we review the state of the art and current trends using CS-based calcium phosphate composites, and especially HAp.

Calcium phosphates

Calcium phosphates have been demonstrated to be the ideal biomaterials for bone implantation during the last 30 years, because of being biocompatible, bioactive and bone-conductive. The development of calcium phosphate-based bioceramics for clinical application began in the 1970s, due to the failure of other previously used materials such as: steel, cobalt alloys and poly (methyl polyacrylate).

The study of calcium phosphate-based ceramics has focused on composites of the tertiary system CaO-P_2O_5-H_2O, mainly hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and tricalcium phosphate [$Ca_3(PO_4)_2$], due to its increased bioactive potential shown in several investigations [8-10].

Among them is Coralina. This is a biomaterial obtained from white corals of the *Porites porites* family which are very common in the Cuban insular shelf, and which bears a crosslinked tridimensional structure quite similar to the human bone trabecula. It is produced as big blocks to fill cavities and in small pieces to replace bone fragments. More than 20 000 patients have been reported as receiving implants of porous HAp with a 97% of bone integration. Only 3.2% of those implants were considered as failures, mainly due to postoperative immediate sepsis requiring biomaterial removal, tumor relapse, and mobility of implants with poor osteosynthesis, and other causes but not to the implanted biomaterial. Coralina application in these specialties allowed treatment and correction of endobucal and facial bone defects, with excellent aesthetic and clinical results [6].

There is also Apafil, a ceramic granulate of highly pure and dense synthetic HAp of different size granules. It has been successfully applied as filling material for periodontal and periapical defects of pathological or traumatic origin, in cystic cavities, to fix intra-osseous dental implants, for filling of autogenous dental transplants cavities and alveolar cavities, to remodel the alveolar edge and to recover the dental pulp, and in rehabilitating tooth root lesions and craniotomies. The preclinical and clinical experiences have demonstrated that Apafil-based HAp ceramics are biocompatible, bioactive, biologically stable and bone-conductive [11, 12].

Bioceramics are only available as blocks or granules, preconditioning some limitations. Granules, for example, are used in small implants, in non-load bearing areas as the middle ear, or in dental implants as coating for the metals used as reinforcement, since bioceramics are rigid and fragile. But it has been proven that these granulated materials are able to migrate to the neighboring sites, increasing the frequency of complications or even causing surgery failure. At the same time, block bioceramics not always fit properly to the treated defect [13-15], and in those few cases where a proper adaptation of the implant is achieved, there is a potential risk of failure due to its very slow resorption kinetics and poor mechanical strength which could result in a short-to-long term fracture.

According to these reasons, partially bioresorbable alternatives have been explored, more similar to the bone tissue to be replaced and serving as scaffold for osteogenesis at the same time that implant resorption occurs. In this sense, partially resorbable ceramics of β-TCP have been developed, obtaining macroporous granules by using an oxygen peroxide solution and yeast powder as porogenic agents [16].

Nonetheless, the bone natural mineral component is mainly non-stoichiometric HAp (of Ca/P ratio other than 1.67) which differs from the stoichiometric

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HAp not only in the chemical composition but also in crystallinity and the specific surface area, these two properties making it more reactive in a biological environment. Some authors mentioned that HAp biodegradation inversely correlates with the Ca/P ratio. In other words, the calcium-deficient HAp is more biodegradable than the stoichiometric HAp due to a low crystallinity [17]. The Ca/P ratios of some calcium phosphates used for biomedical applications are shown in table 1.

It was recently reported the development of a calcium-deficient HAp (Ca/P ratio lower than 1.67) of nano-structured morphology, which was doped with silicate ions to imitate the biological apatite, which was able to increase the solubility and resorption capacity *in vivo* of the composite materials it was designed for, also increasing their bone-conductivity and bioactivity [18].

Simultaneously, a whole range of possibilities arose with the discovery of CPCs in the 1980s for using calcium phosphates in bone tissue regenerative applications. In general, CPCs are composed of two phases, one solid and one liquid, which form a paste at mixing and harden under physiological conditions once implanted by injection. For this reason, they can be used in low invasive surgical procedures.

Some recent studies focused on creating acrylic bone cements modified with HAp micro and nanoparticles, more resistant to compression and traction than the same type of materials used for more than 40 years in orthopedics to fix the articular prosthesis to the bone [19].

Another current trend for preparing bone implant calcium phosphates comprises adding macroporosity. This relevant property supports the fast cellular colonization of the implanted material (Figure 1) by accelerating the vascularization process and, therefore, a satisfactory repair of the bone defect [20, 21], this property having to be tightly controlled for the bioresorption of the bone implant. Some authors consider as macroporous materials those having pore diameters from 50 to 250 µm. However, Kawachi *et al.* advise that the ideal pore diameter must be above 100 µm for bioceramics [22].

The methods used for pore formation can be divided in two major groups: emulsion formation, to generate pores during the hardening process but followed by a thermal procedure to achieve the required porosity, and the addition of highly soluble and non-toxic crystalline substances, adequate to produce the required porosity during powder mixing. This last method requires dissolving the additives once the cement has been applied and hardened onto the bone defect.

The use of mannitol, sucrose, and frozen particles of a sodium phosphate solution containing surfactant molecules are the most established techniques to induce porosity in CPCs. All of them support the formation of macropores on CPCs, but of deficient crosslinking. To solve this problem, some authors have implemented pore formation by *in situ* gas formation because of adding $CaCO_3$ to the cement powder, followed by a reaction while mixing [23]. Recently, a technique was developed for macropore formation in CPCs by using egg albumin as foamy agent during cement hardening [16]. The same authors ran several experiments to obtain macroporous HAp scaffolds from CPCs with the aid of hydrogen peroxide as foamy agent, producing cements with crosslinked macroporosity of pore wider than 200 µm, the best report on CPCs so far [24].

Table 1. Ca/P ratio of some of the calcium phosphates used in medical applications

Name	Formula	Ca/P
Tetracalcium phosphate	$Ca_4(PO_4)_2O$	2.0
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	1.67
Calcium deficient hydroxyapatite	$Ca_9(HPO_4)(PO_4)_5(OH)$	< 1.67
Tricalcium phosphate (α,β)	$Ca_3(PO_4)_2$	1.5
Dicalcium phosphate dihydrated (Brushita)	$CaHPO_4.2H_2O$	1.0

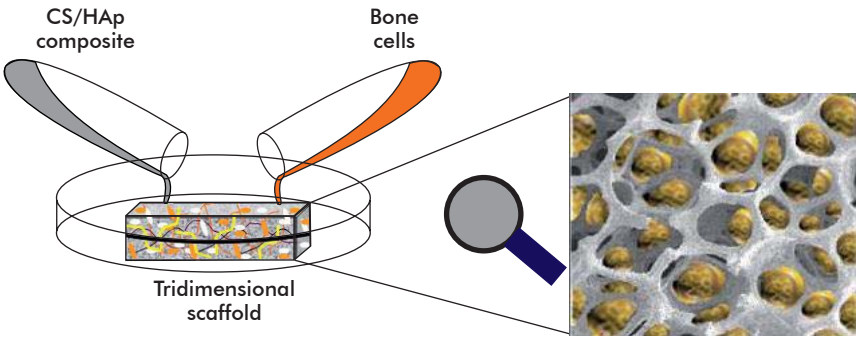

Figure 1. Osteoblast colonization of tridimensional scaffolds.

Nevertheless, it has been demonstrated that CPCs still have a very slow resorption kinetic and a poor mechanical strength in spite of its described advantageous properties, both considered disadvantageous due to the weakness of the material as compared to the surrounding tissue [25]. These limitations have led to investigate on biopolymer-based calcium phosphate composites, bearing properties which can be incorporated to the resulting material.

Biopolymers

Polymeric materials are widely applied in the field of medical implantology, due to their similarity to the physical-chemical properties of the living tissues which are mainly composed of natural polymers or biopolymers such as proteins and polysaccharides [26]. These materials have been used for drug controlled release and tissue engineering, because of being biocompatible, non-toxic and biodegradable at physiological conditions, and showing a variable *in vivo* resorption kinetic according to the properties of the given macromolecule.

As mentioned above, there is a current trend on using biopolymers to formulate materials for periodontal, maxillofacial and bone tissue engineering applications. The physical properties of the polymeric materials are similar to those of the organic phase present in bone tissues, in spite of having certain limitations hampering its application in those body structures subjected to high and cyclic loads and tensions, such as hip and knees. These properties allow establishing bone structures through their matrices, supporting the fastest osteointegration, and resorption of natural polymer composite materials.

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Collagen, jelly, alginate, chitin and CS are among the most widely studied biopolymers. CS has been extensively reported as a versatile biopolymer, due to its excellent properties and diverse natural sources, being considered by some groups as the natural polymer of the 21st century and with several applications in biotechnology, biomedicine and recently in tissue engineering [27].

Chitosan

Chitosan [(1→4)-2-amino-2-desoxy-β-D-glucan] (Figure 2) is industrially produced by extensive deacetylation of chitin [(1→4)-2-acetamide-2-desoxy-*β*-D-glucan], a polysaccharide widely found in the animal kingdom and the second in abundance after cellulose what makes it an important renewable resource.

Chitosan is composed of two types of structural units randomly distributed (Bernoulli distribution) along the chain: N-acetyl-D-glucosamine (A) and D-glucosamine (D), linked together by glycosidic type *β* (1→4) bonds. In its crystalline form, CS is normally insoluble in neutral water solutions, but soluble in diluted acidic solutions (pH< 6.0) where glucosamine amino free groups become protonated [28].

Depending on the source and preparation procedure, CS molecular weight can range from 300 to 1000 kDa with a deacetylation degree (DD) of 60 to 95%. Chitosan has been demonstrated to be biocompatible and biodegradable, because of promoting cell adhesion and being reabsorbed by means of hydrolysis by enzymes present in the physiological fluids [29, 30].

It can be considered a structural analog of glycosaminoglycans (GAG)[31, 32], anionic polysaccharides generally bound to proteins by electrostatic interactions to form proteoglycans. The last ones play a relevant function in organizing and functioning of the extracellular matrix in human tissues [33].

Chitosan- and GAG-based materials, said CS and chondroitin sulphate, have shown adequate cytocompatibylity for bone regeneration, successfully used for cartilage repair and as artificial skin components to treat dermal lesions. Studies comparing different materials composed of CS and alginate, or polylactic acid (PLA), for articular tissue engineering, showed that CS could increase adhesion and cell proliferation *in vitro* and support a better biosynthetic activity than alginate and PLA [34].

Lee *et al.* [35] obtained a scaffold formulated with CS, GAG, collagen and loaded with tumor growth factor β1 (TGF-β1) for its controlled release to promote articular regeneration. They improved the mechanical properties and stability of the collagen macromolecules' crosslinking by adding CS, also inhibiting the action of collagenases. Aimin *et al.* [36] demonstrated the antibacterial activity of CS, a subject extensively referenced in the consulted literature [37]. They evaluated CS in experimentally-induced osteomyelitis, showing a reduction in the spread of a *Staphylococcus aureus* infection. Sano *et al.* [38] studied the influence of CS DD and molecular weight on the inhibition of *Streptococcus sobrinus* 6715 in saliva treated with hydroxyapatite, and demonstrated the potential antiplatelet effect of high molecular weight CS (5-6 kDa) and intermediate DD (50-60%) [39].

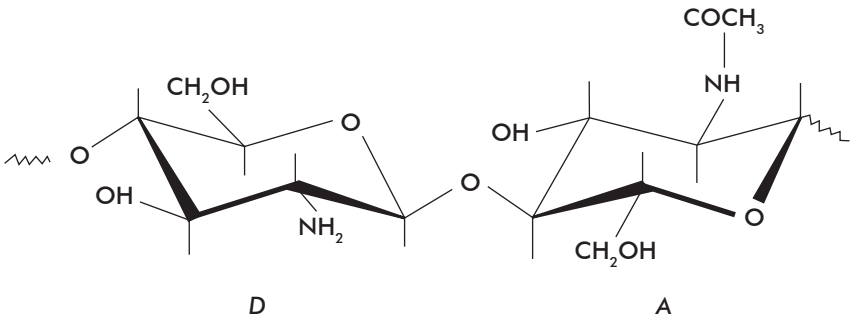

Figure 2. Repetitive units on the chemical structure of CS. A) N-acetyl-D-glucosamine, and D-glucosamine (D).

Chitosan biodegradability is another essential factor to consider when selecting biopolymers for tissue engineering. Lysozyme is the enzyme present in biological fluids responsible for CS degradation *in vivo*.

Lysozyme hydrolyzes the β-1,4 de N-acetylglucosamine bonds close to a glucosamine residue. By these means, lysozyme action on high DD CS (> 80%) is weak, with no significant changes in the molecular weight of the hydrolyzed CS over time. This results in long polymer degradation periods, delaying in months the *in vivo* resorption of the CS-based material [40]. Mao *et al.* [41] observed a direct correlation between CS DD and cell adhesion. A fast biodegradation could be achieved by using low DD CS, but limiting cell adhesion. Therefore, the adequate selection of DD for the CS to be used is critical to develop tridimensional scaffolds and control its biodegradation and biocompatibility.

The abovementioned properties of CS [42], together with its demonstrated healing and hemostatic properties, its plasticity for presentation (microspheres, membranes and bidimensional films, or in tridimensional scaffolds), flexibility, mucoadhesivity and wettability, also its facility to promote bone formation by cellular osteogenesis at the required site, among other desired properties [43], support using CS as an excellent polymeric candidate in orthopedical applications, especially for bone tissue engineering [44]. Nevertheless, CS is not bioactive as biopolymer and show poor mechanical properties.

All these explains the growing interest on combining the bioactivity and biodegradability of inorganic materials together with the rest of CS properties to obtain new compound materials (composites) of improved mechanical characteristics also favorable for bone tissue engineering [45].

Preparation of CS and HAp composites: main formulations, procedures to obtain them and applications

Calcium phosphate composites with CS matrices were extensively investigated in the last decade for structural purposes, specifically for orthopedics and maxillofacial surgery [46], either to fill bone defects, to increase the alveolar edge, middle ear implants, fusion of spine vertebrae or to coat metallic prosthesis, due to a proper filling-matrix integration [47]. Those hybrid materials have been prepared by different methods, as shown in table 2.

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Chitosan and HAp simple mixing method

The method of simple Hap mixing with CS is one of the most common methods used to prepare composites, especially tridimensional scaffolds for tissue engineering [48]. The technique consists in mixing the previously prepared ceramic powder with a CS solution to generate a suspension as homogeneous as possible. Scaffolds are generated from this mix as porous sponges by freezing and lyophilization [49, 50]. Murugan *et al.* [51], for example, used this approach to develop a simple procedure, by using the wet way, to generate nanostructured compound systems (nanocomposites) of HAp and CS in two steps: HAp precipitation in alkaline medium (pH 10.0 with NH_4OH) starting from $CaCl_2$ and $(NH_4)_2HPO_4$ as precursor salts and further mixing of nanometric HAp with solutions at different CS concentrations in acetic acid at a fixed temperature.

A similar procedure was used by Finisie *et al.* [52] to generate HAp and CS composites in pills. They started from a paste made up of a mix of HAp, aluminum and CS at different proportions. Due to aluminum toxicity, they transformed it onto sodium aluminate by using a concentrated solution of sodium hydroxide, resulting in the formation of pores wider than 100 µm which were generated by the hydrogen gas released by the reaction.

Incorporation of CS to CPCs

The incorporation of CS to CPCs (Figure 3) is another very promising way to obtain composite materials, since it improves injectability, the degradation rate, hardening properties and mechanical performance of the resulting materials [53]. In this case, a suspension of the solid fraction is mostly obtained in the polymeric matrix with pH near 6 where there is a fraction of CS protonated amino groups enough for solubilization, aiding to include CS into the liquid fraction of the cement. Afterwards, an injectable viscoelastic paste is prepared, which contains CS incorporated in the liquid fraction of the cement and the solid fraction HAp precursors. Once injected, the paste hardens due to the rise of pH to physiological 7.4 and the subsequent neutralization of the amino groups. In this process, the inorganic load becomes physically entrapped (*in situ* hardening) within the gel matrix in 5 to 8 min, an interval shorter than for control CPCs (61.7 min.) which are devoid of CS [54]. The resulting materials also show improved mechanical properties, as resistance to compression of 15 to 25 MPa, higher than the same parameter for the CPC alone (10.4 MPa) [55].

Taking advantage of these facilities, some investigations have focused on CS-CPC composites [56-62] as materials with potential application in bone tissue engineering and for controlled release of therapeutic agents, showing very promising results in periodontal bone defect regeneration [63]. In this sense, a vast experience has accumulated on the synthesis and characterization of compound materials by using calcium phosphates as filling materials (β-TCP and HAp, treated superficially or not), natural polymers (CS, sodium alginate), and acrylics (mainly poly acrylic acid, polyacrylamide and poly(methyl methacrylate), poly(2-hydroxyethyl methacrylate)) to restore bone tissue and as potential controlled release systems for active ingredients [64, 65]

Table 2. Methods to obtain CS/HAp composites and their formulations

Method	Formulations
Mixing of both components, CS and hydroxyapatite	Membranes [118], films[119] and scaffolds [120, 121]
Incorporation of CS to calcium phosphate cements (CPCs)	Macroporous supports [122] and injectable materials [123]
Coating of hydroxyapatite with CS	Multilayered films [68], plaques [124], porous scaffolds [125] and others [77]
In situ precipitation and co-precipitation of the inorganic bioactive component inside the polymeric matrix	Films [126], particles [127], porous supports [128] and others [129]

Coating method

Calcium phosphate composites can be obtained as coating by using electrochemical and electrophoretic procedures, which favor the deposition of HAp and CS particles. This technique commonly known as electrochemical deposition allows preparing hybrid materials at room temperature, which is highly favorable considering the final biomedical application.

Recently, Pang *et al.* [66] developed a method of layered electrophoretic co-deposition of the CS-HAp composite for protecting steel-made materials getting into contact with physiological solutions. To modify the surface of shape memory materials made up of nickel-titanium alloys and protect them from corrosion, Sun *et al.* [67] prepared composites of heparin, HAp and bioglass in a CS polymeric matrix as films, by cathodic electrophoretic deposition. Redepenning *et al.* [69] also prepared HAp and CS composites by an electrochemical method, starting from brushita and CS in alkaline medium, to coat titanium materials [68].

Additionally, some authors have studied the remarkable properties of this technique by using biomaterials as Biovetir II, coated with nanostructured CS-HAp composites to improve the implant interaction with cells and its fixation to the subcutaneous tissue [69], as well as for middle ear reconstruction [70].

In situ precipitation method

In spite of the overwhelming advantages of the abovementioned methods, it has been demonstrated that

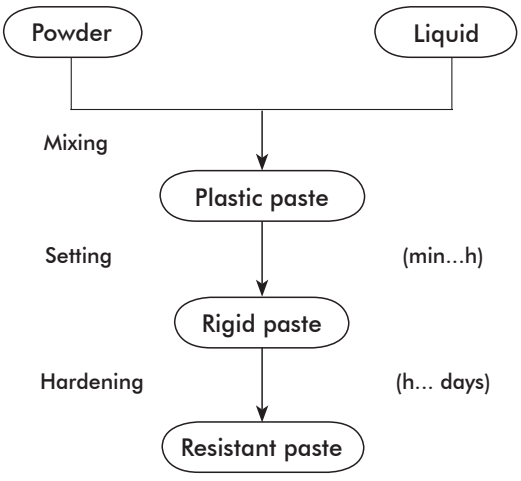

Figure 3. Preparation of a cement material.

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compound systems prepared by mixing techniques, which incorporates CS into CPC, and those coating HAp particles with CS solutions are frequently non-homogeneous at microscopic level because of the hard to obtain uniform distribution of the inorganic components in the womb of the polymeric matrix. These can generate practical inconveniences, since the lack of homogeneity can induce inflammatory symptoms by forming voluminous fibrotic capsules of dense or granulated connective tissue. This originates a strong rejection of the implanted material or changes in the area surrounding the prosthesis.

In that sense, the method of *in situ* precipitation of the bioactive inorganic component inside the polymeric matrix, as referred in several publications as biomimetic or bio-inspired method [71] could be more attractive to prepare this type of composites by permitting to generate much more homogeneous systems.

This procedure is nature-inspired in bone, dentin, egg and mollusk shells, etc., showing a perfect combination of the structure and composition characteristics responsible for the micro and macro-properties. In situ precipitation of calcium phosphates allows controlling the internal architecture (structure) and the chemical composition of the resulting materials.

This procedure is based on obtaining nanostructured and crystalline composites of the biologically active component (calcium phosphate), homogeneously disperse in the polymeric matrix (CS) to mimic the biological apatite of the bone tissue. This supports its application in bone tissue engineering, specifically in targeted bone regeneration [72], and controlled release of drugs [73] and biologically active molecules [74].

These materials have being traditionally prepared by several methods. Yamaguchi *et al.* [75] have developed a single-step co-precipitation methodology by dripping CS in H_3PO_4 solution on a calcium hydroxide suspension. Fan *et al.* used CS biomineralization in solid phase by soaking in a simulated biological fluid (SBF) [76]. Redepenning *et al.* mixed a solution of the biopolymer with calcium phosphate inorganic precursors used as filling, followed by composite precipitation as hydrogel or isolated particles [77]. Hu *et al.* [78] reported an interesting methodology, using CS hydrogels for mineralizing it via *in situ* hybridization and controlling the process by ionic diffusion. Rusu *et al.* [79] studied the kinetics of composite formation for CS and HAp composites prepared by co-precipitation. In that process, pH of the CHI solution and saline precursors ($CaCl_2$ and NaH_2PO_4) were gradually increased by adding a sodium hydroxide solution, and composites of a high degree of structural organization were obtained by using soluble saline precursors (equation 1).

$$CaHPO_{4\,(ac)} + 12\ OH^-_{(ac)} \longrightarrow Ca_{10}(PO_4)_6(OH)_{2\,(s)} + 10\ H_2O + 4\ PO_4^{3-}$$

The same group suggested the use of salts and weak acids as precursors of calcium and phosphate ions, instead of strong acids or bases to avoid their potential destructive effect on the polymeric chains of CS during composite preparation. Chang *et al.* [80] reported a similar procedure to prepare HAp and gelatine nanocomposites, with Ca^{2+} and PO_4^{3-} ions concentrations controlled by diffusion, through dissolution and co-precipitation processes.

These *in situ* production methods have supported an adequate incorporation of inorganic fillings inside composites' structures, either at micro or nanometric scales. Noteworthy, each of the methods referred generates a particular type of CS and HAp composite material, with specific structural properties.

Considering that the mineral phase of the bone is composed of non-stoichiometric HAp (Ca/P ratio < 1.67), it is convenient to simulate the most biological apatite with the inorganic component on these composites. For this purpose, Peña *et al.* [81] mixed brushita powder ($CaHPO_4{\cdot}2H_2O$) with a CS citric acid solution, and keeping the solution alkaline by adding sodium hydroxide. The use of citric acid dissolutions as solvent is questionable in spite of the similarity of the apatite phase to that of the final composite, since it inhibits HAp transformation while nucleating amorphous calcium phosphates. Besides, there was also mentioned in the literature that citrate ligands can affect crystal and cell size because of including carboxyl groups [82].

We have prepared in our lab composites of CS and calcium-deficient HAp starting from acetic acid solutions of HAp precursor salts (calcium acetate and $NaH_2PO_4{\cdot}H_2O$) and CS, with control of the calcium phosphate phase to optimize hydrolysis conditions [83]. Equations 2 and 3 describe the formation steps of the non-stoichiometric apatite obtained *in situ*, within the polymeric CS matrix.

$$CaAc_{2(ac)} + NaH_2PO_{(ac)} + 2H_2O \xrightarrow{CHI} CaHPO_4{\cdot}2H_2O_{(s)} + 2HAc_{(ac)}$$

$$(10\text{-}x)CaHPO_4{\cdot}2H_2O_{(s)} + \frac{x}{3}Na_3PO_{4(ac)} + xH_2CO_{3(ac)} \xrightarrow{CHI} Ca_{10-x}Na_x(PO_4)_{6x-4}(CO_3)x(OH)_{2(s)} + \left[\frac{12+x}{3}\right] H_3PO_{4\,(ac)} + (18 - 2x)\ H_2O$$

Optical micrographs as those shown in figure 4 correspond to the inorganic phase crystals obtained on each phase according to equations 2 and 3, prior to (calcium hydrogen phosphate; $CaHPO_4{\cdot}2H_2O$ or DCPD) and after (calcium-deficient HAp precipitated in the absence and presence of CS) the hydrolysis with Na_3PO_4.

A morphological examination shows that in the first step, the inorganic phase precipitating under the reaction conditions used corresponds to the typical flattened crystals of brushita ($CaHPO_4{\cdot}2H_2O$). However, after hydrolysis with Na_3PO_4, much smaller crystals were obtained by formation of the expected apatite phase (calcium-deficient HAp), achieving a complete transformation of one crystalline phase into the other.

We have recently prepared via *in situ* composites of CS with non-stoichiometric HAp incorporating silicate ions to generate a material of higher osteointegration to the biological system [84]. The load of antibiotics and other active principles (for example, growth factors) into these types of tridimensional matrices could be a way to increase cell proliferation, with faster and more efficient formation and mineralization of the bone tissue.

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Physical-chemical characterization of composite systems of CS and HAp

The physical-chemical properties of CS and HAp are highly similar to those of the bone tissue [85]. The biodegradable, biocompatible and bone-conductive performance of these composite materials is very relevant for future medical applications [86].

In general, the inorganic reinforcement of this type of composites should provide simultaneously bioactivity together with resistance and rigidity to the resulting material, the polymeric matrix functioning as filling. However, it has been demonstrated that the properties of the generated material, the biological properties included, can be improved by incorporating CS [87].

Biodegradation

Biodegradation or the changes affecting the implanted device when exposed to the natural physiological or simulated medium is critical and has been extensively addressed in the literature [88, 89]. Several factors can be involved: physical-chemical dissolution, physical breakdown of bigger molecules into smaller ones by biological agents as enzymes [90] what decreases pH at the site [91], or the properties of the polymeric matrix used, among others [92, 93].

In the same line of evidence, Yang *et al.* demonstrated that the degradation kinetics can be controlled by selecting the N-acetylated CS derivative, also accelerating its resorption (with lower DD values) due to the preferential action of lysozyme on the glycosidic bonds between the acetylated units of the biopolymer [93]. Nevertheless, Freier *et al.* observed that lower DD CS films induce poor cell adhesion [88]. Similarly, Chatelet *et al.* showed that fibroblasts did not proliferate on CS films regardless of its DD value, due to a tight anchoring of these cells onto the material that prevents its further proliferation [94]. Putting all together, it suggests that biological properties of CS-based composite materials not only depend on the acetylation/deacetylation balance of the natural polymer, but also on the cell type used to promote adhesion and proliferation on the given substrate.

A method to evaluate the degradation rate comprises assessing the release of Ca^{2+} ions from composites submerged into a physiological medium. Results show an increasingly faster degradation rate by increasing composites CS content. The hydrophilic nature of the CS polymeric matrix generates a low crystallinity in composites, subsequently rising the solubility of the system, the dissolution speed and the release of calcium ions to the medium. This supports a gradual rise of bioresorption resulting from the increased biopolymer content within the composites [95].

Morphology

The final morphology of composites is another significant property influencing the functionality of these materials. It is affected by reaction conditions, such as: concentration, temperature, pH, 3D conformation, time and filling-matrix interaction.

Generally, composite morphology can widely vary depending on the geometric versatility provided by the polymeric matrix (films, micro and nanoparticles, membranes, cements, etc.) and the intended application. Normally, morphology, size and distribution are measured by optical and/or scanning and transmission electron microscopy techniques. A relevant aspect is the apatite phase obtained on the surface of the composites, which frequently appears as globular aggregates or clusters of tiny crystals with different characteristics depending on the predominant inorganic component in the given composite [96]. For example, β-TCP is presented as prism-like particles, the octocalcium phosphate as small clustered plaques, quite similar to the non-stoichiometric HAp, and DCPD as wide, flattened crystals of great size, and calcium deficient HAp generally appearing as thin and small needles projected from a central point (Figure 4) [97].

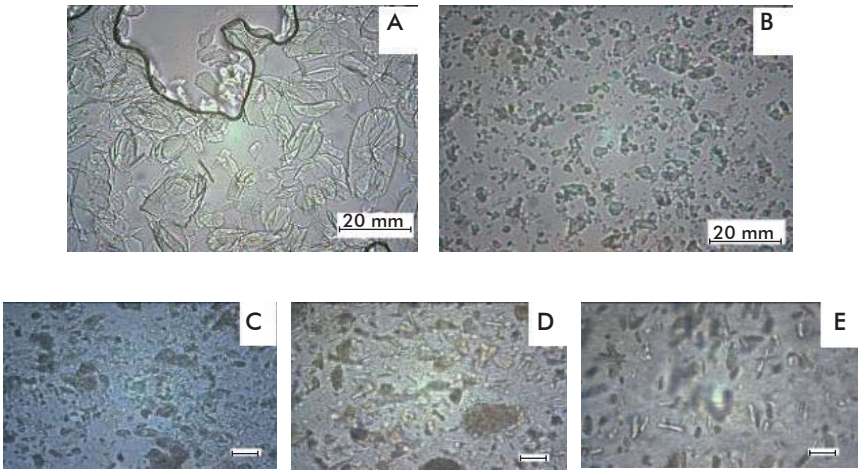

Figure 4. Optical micrographs (500 x) of inorganic phase crystals. A) Before Na3PO4 hydrolysis (CaHPO4·2H2O, DCPD), B) after hydrolysis (calcium deficient HAp) and in the absence of CS/HAp composites, C) 20:80 CS/HAp ratio, D) 50:50 CS/HAp and E) 80:20 CS/HAp.

Composition

Composition of CS/HAp is frequently studied by infrared spectroscopy to identify the main functional groups and the presence or absence of carbonate ions. Crystallinity and the inorganic phases present are assessed by X-ray diffraction, and Ca and P content determined by energy dispersive spectroscopy, while the composition of the system is evaluated by thermogravimetry and differential thermal analysis [98].

The individual components of CS calcium phosphate composites show a stable physical-chemical identity; in other words, they are neither dissolved nor completely fused one into the other. Thus, there can be independently identified the inorganic phase and the polymeric matrix. Nevertheless, they can interact through the reactive amino groups on CS and the phosphate and hydroxyl groups and calcium ions on the HAp [99].

Biological assays of CS/HAp composites

Biocompatibility is the property of any material determining its fitness to a specific application without inducing allergic, immunitary or any other type of reaction when getting into contact with living tissues [100]. Calcium phosphate and CS composites have shown to be biocompatible in biological assays with different cell types [101].

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In another experimental setting, HAp composites based on CS and alginate and soaked with drugs showed effective as local delivery systems *in vitro* [102]. Similarly, the *in vitro* encapsulation within CS/HAp microspheres and bone-conductivity of human periodontal ligament fibroblasts were assessed, providing an adequate osteogenic differentiation [103].

Additionally, bioactivity is the ability to support the formation of a biologically active carboHAp layer onto its surface, facilitating the interface junction with the bone tissue and guaranteeing a close attachment to the bone without an interposed fibrotic capsule. This supports the efficient material-bone integration, allowing the presence of bone cells as required for a successful regeneration process on the material´s surface [104]. Hence, the inorganic phase to be integrated should be chemically and structurally similar to the mineral phase of the bone. In this sense, the excellent bioactivity of HAp [105] and the presence of the N-acetylglycosamine in CS (GAG analog) stimulate bone growth [106].

Biodegradability is another relevant property for tissue engineering systems, also considered for its selection and with CS positively contributing to it. By these means, a dynamic process of formation and resorption of bone tissues is established, where these materials serve as scaffolds for tissue infiltration and replacement, simultaneously stimulating cell growth within [107].

Recently, porous tridimensional scaffolds of calcium phosphate and CS composites were generated for bone tissue engineering [108, 109]. These systems, obtained from CPCs and CS derivatives, tricalcium phosphate/CS, alginate/HAp and CS/HAp [110] supported fast osteoblast differentiation and growth (Figure 1), with osteogenic effects [111]. Zhao *et al.* [112] prepared a 3D lattice of HAp/CS/gelatin (a composite with a structure similar to that of the human bone) by *in situ* precipitation and intended for bone tissue engineering, to induce favorable adhesion, growth and osteogenic differentiation of cultured human mesenchymal cells.

Additionally, the group of Zhang *et al.* [113] has published several works where they obtained calcium/CS porous scaffolds, alternatively incorporating bioglass for human MG63 osteoblast cell culture. Only osteoblast cells were able to proliferate or differentiate within all the composite materials (phenotypic proliferation). The use of CS scaffolds together with calcium phosphate bioglass avoid the fast degradation in the physiological medium, this process releasing acid byproducts which could affect the osteoblast differentiation [114].

Also Zhao *et al.* [115] developed hybrid biomimetic materials of HAp/CS/gelatin to analyze cell behavior in rats. Results showed cell proliferation, *de novo* formation of bone tissue and mineralization of the obtained scaffolds in only 3 weeks [116].

These investigations demonstrate the feasible use of calcium phosphate/CS tridimensional matrices as adequate systems for scaffolds and implants in bone tissue engineering procedures [117]. The proper selection of the components for the system to be designed will be determined by the final application of the scaffold.

Conclusions

Ceramics of calcium phosphates as HAp, osseous CPCs and β-TCP, are biocompatible, bioactive and osteoconductive, these properties making them excellent materials for orthopedics. Nevertheless, their availability as granules or blocks, the mechanical weakness of CPCs and the slow resorption or degradation *in vivo* of all these materials limit their application and the useful lifespan of the material with adequate characteristics, both short and long term in the host organism. Composites formulations based on natural polymers as CS and the range of preparation methods available allowed obtaining materials of improved and fitted design, of increased strength and improved bone-conductivity, homogeneity, bioactivity, biodegradability and similar to the bone tissue to be repaired or replace. All these widen their application in the fields of orthopedics and traumatology, more than precedent materials. Current trends tend to develop composite tridimensional scaffolds of improved properties, starting from inorganic materials of higher biodegradability and bioactivity (for example, Si-doped non-stoichiometric HAp) and incorporating antibiotics and other active principles (*e.g.*, growth factors) into the matrix of CS or other polymer to promote an adequate cell proliferation, and a faster and more efficient formation and mineralization of the injured bone tissue.

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