CHAPTER

11 New insights into nanohydroxyapatite/chitosan nanocomposites for bone tissue regeneration

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11.1 INTRODUCTION

A great interest has been given to synthetic and stoichiometric hydroxyapatite (HAp) as a biomaterial due to its similarity with the mineral phase found in hard tissues (Levengood and Zhang, 2014). It possesses exceptional biocompatibility (Chen et al., 2007) and bioactivity (Hossein Fathi et al., 2009; Fathi et al., 2008) with respect to bone cells and surrounding tissues, which makes it a suitable material for the replacement of small parts of bone, filling of cavities in dentistry, and coating of metallic implants (Ferraz et al., 2004). Additionally, this bioceramic has a high capacity of ad- and absorption, making it an excellent candidate for drug delivery applications, such as long-acting drugs for anticancer treatment of bone tumors (Itokazu et al., 1998; Bose and Tarafder, 2012).

Bones are not only the frame that supports the human body, allowing its mobility and protecting it against injury, but also the storehouse for minerals that are essential for the functioning of other life-sustaining systems in the body. For these reasons, healthy bones are critically important for the overall health and quality of life. Unfortunately, bone is the most frequently transplanted tissue after blood transfusions. The clinical need to replace, restore, or regenerate bone tissue comprises the fields of dental, maxillofacial, orthopedic, spinal, and cranial surgery, being osteoporosis the most important cause of fracture in the elderly (Brydone et al., 2010; Campana et al., 2014; Services, U.S.D.o.H.a.H., 2004).

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As a matter of fact, the International Osteoporosis Foundation reported in 2010 an estimation of 3.5 million new fractures in the European Union caused by osteoporosis, representing an economic burden of approximately 37 billion EUR (Svedbom et al., 2013).

Medical solutions for bone repair and regeneration are still a major challenge. Even though they present serious drawbacks, biologically derived bone grafts have been implanted for many years, as temporary or permanent prostheses, and are still today available in the market at great scale. Due to the numerous disadvantages of biological grafts, current efforts are focused on the development of manmade materials that, in comparison with orthodox treatments, have great potential to improve the regeneration of bone or its replacement when damaged, with the advantage that they are easily available and might be processed and modified to suit specific needs (Brydone et al., 2010; Bose et al., 2012). In this context, the development of HAp-based nanocomposite materials is very promising not only in terms of market opportunities, but also due to the contribution they could impart to regenerative medicine, particularly for hard tissue regeneration.

The first attempt to produce an implantable polymer/Ca-P composite was carried out in 1981 and consisted in the development of HAp/polyethylene formulations by pioneer Prof. William Bonfield and his colleagues (Bonfield et al., 1981). Ever since, many studies have been conducted in this field, including the use of FDA-approved, synthetic-biodegradable polymers such as poly(L-lactic acid) (PLA; Wei and Ma, 2004), poly(glycolic acid) (PGA; Agrawal et al., 1995), and their copolymers (PLGA), with applications as drug carriers, bone fixation screws, plates, resorbable sutures, among others (Agrawal and Ray, 2001; Dorozhkin, 2011).

More recently, the incorporation of nanohydroxyapatite (n-HAp) into natural polymers is recognized as one of the most viable approaches to produce materials mimicking natural bone. Besides the expected biodegradability, natural polymers can be biologically active promoting better interactions with cells and, therefore, excellent cell adhesion and growth (O'Brien, 2011). In particular, the combination of n-HAp with chitosan (CS) has been of great interest for the production of nonload-bearing bone grafts, given the advantageous properties of CS that allows the manufacturing of nanohydroxyapatite/CS (n-HAp/CS) materials with improved properties. Among other features, CS promotes osteoblasts adhesion and proliferation, possesses antimicrobial properties, and can be processed using mild conditions and shaped into different forms (Costa-Pinto et al., 2011). However, besides the many studies proving its suitability for tissue regeneration, n-HAp/CS materials have not yet reached the market at great scale.

This chapter is a compilation of fundamentals in bone tissue engineering, focusing on the recent advances on the use of HAp and CS as biomaterials for bone regeneration, and progresses in what concerns the fabrication of HAp/CS nanocomposite materials.

11.2 OVERVIEW OF BONE BIOLOGY

Natural bone is a complex hybrid material with extracellular matrix (ECM) composed of approximately 60%−70% of an inorganic mineral, precipitated onto 20%−30% of an organic matrix, and a small portion of water (Levengood and Zhang, 2014; Amit et al., 2006). Structurally, it is highly organized in a hierarchical manner, from the nano- to the macroscale, as shown in Fig. 11.1. At the nanoscale, the mineral phase consists of calcium phosphate, plate-like nanocrystals, of 2−6 nm thickness, 30−50 nm width, and 60−100 nm length, chemically and structurally very similar to synthetic n-HAp (Levengood and Zhang, 2014; Amit et al., 2006) (see Table 11.1).

At the microscale, bone can be divided in two categories based on density: cortical and cancellous bone. Cortical or compact tissue is very dense, with a porosity of 3%−5%, and forms the outer layer of bone, providing support and protection. The less-dense inner part is constituted by cancellous bone, also known as trabecular or spongy-like bone; it is highly porous, with 30%−90% porosity, and contains the bone marrow (Levengood and Zhang, 2014; Bose and Tarafder, 2012; Costa-Pinto et al., 2011; Burr and Allen, 2013). Together, the cortical and trabecular tissues form the skeletal element of bone. However, bone is also a very dynamic tissue that constantly experiences modeling and remodeling processes as a consequence of mechanical and metabolic changes.

Five primary cells dictate bone formation and remodeling: mesenchymal stem cells (MSCs), osteoblasts, osteocytes, osteoclasts, and bone lining cells. MSCs are found in the bone marrow and in the periosteum, the fibrous layer on the outside surface of bone. They are multipotent stromal cells that can differentiate into osteoblasts, the single-nuclei cells in charge of bone matrix protein secretion and bone mineralization. After completing their main function of synthesizing bone matrix, osteoblasts undergo three different transformations: they remain entrapped in bone as osteocytes, they flatten into bone lining cells, and the remnants suffer apoptosis.

Osteocytes are the most abundant cells present in bone, representing more than 90% of cells within the bone matrix and surfaces, and their main function is the coordination of osteoblasts and osteoclasts functions in response to mechanical and hormonal signals. Bone lining cells are relatively inactive forms of osteoblasts whose function is to cover the inactive surface of bone; they may recover the ability to synthesize bone matrix and participate in the regulation of calcium exchanges. Finally, osteoclasts are the cells responsible for the resorption of mineralized bone. All these cells work together in a balanced way to maintain the integrity of healthy bone, as well as to regenerate bone that has suffered trauma or disease. In the last case, when the defect size is critical, bone grafts are necessary to assist bone healing (Levengood and Zhang, 2014; Costa-Pinto et al., 2011; Burr and Allen, 2013).

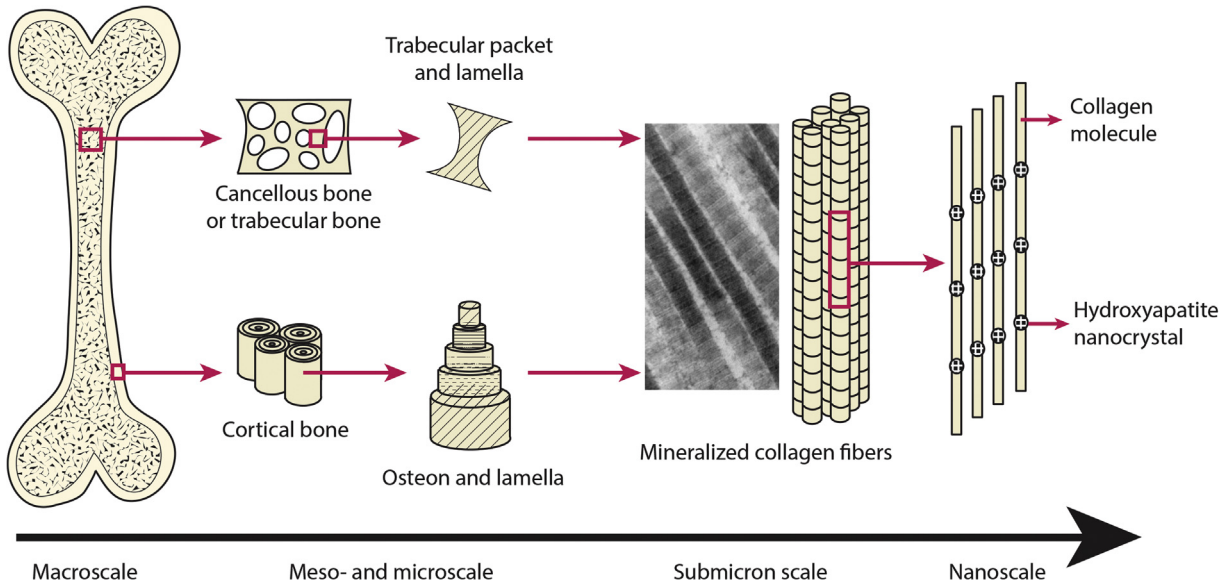

FIGURE 11.1

Hierarchical organization of bone.

Table 11.1 Typical Composition of the Inorganic Phase of Adult Human Calcified Tissues and Hydroxyapatite

Composition	Enamel	Dentin	Bone	Hydroxyapatite
Calcium (wt.%)	36.5	35.1	34.8	39.6
Phosphorus (as P) (wt.%)	17.7	16.9	15.2	18.5
Ca/P (molar ratio)	1.63	1.61	1.71	1.67
Sodium (wt.%)	0.5	0.6	0.9	–
Magnesium (wt.%)	0.44	1.23	0.72	–
Potassium (wt.%)	0.08	0.05	0.03	–
Carbonate (as CO_3^{2-}) (wt.%)	3.5	5.6	7.4	–
Fluoride (wt.%)	0.01	0.06	0.03	–
Chloride (wt.%)	0.30	0.01	0.13	–
Pyrophosphate (as $P_2O_7^{-4}$) (wt.%)	0.022	0.10	0.07	–
Total inorganic (wt.%)	97	70	65	–
Total organic (wt.%)	1.5	20	25	–
Water (wt.%)	1.5	10	10	–

Adapted from Bose, S., Tarafder, S., 2012. Calcium phosphate ceramic systems in growth factor and drug delivery for bone tissue engineering: a review. Acta Biomater., 8 (4), 1401–1421.

11.3 THE IDEAL BONE GRAFT

Considering the compositional, structural, mechanical, and biological complexity of natural bone, the design of an ideal bone graft is not an easy task. The requirements for a graft intended to mimic all features of bone are numerous and diverse, however, there are a few key factors that must be fulfilled.

Based on O'Brien (2011), they can be summarized in terms of biocompatibility, biodegradability, mechanical properties, structural requirements, and manufacturing technology.

11.3.1 BIOCOMPATIBILITY

An indispensable requirement of bone grafts is biocompatibility. This term can be defined as the ability of a material to support normal cellular activity, including molecular signaling systems, without causing any systemic or local effects to the host tissue (Bose et al., 2012). Therefore, the graft should be *osteoconductive*, that is, it should promote cell adhesion on its surface and migration within its pores, cell proliferation, and formation of ECM. In addition, *osteoinductive* properties are necessary, that is, the bone substitute must induce the formation of new bone through the reclusion of progenitor cells and biomolecular signaling (Bose et al., 2012; Ricciardi and Bostrom, 2013). Along with osteoconductive and osteoinductive properties, products of bone graft degradation should be nontoxic and easily excreted by metabolic pathways (Liu and Ma, 2004).

11.3.2 BIODEGRADABILITY

The main objective of a bone graft is to assist tissue regeneration by providing a bridge that will fill critical size bone defects in such a way that it allows the body's own cells to replace, over time, the implanted substitute. Therefore, grafts should degrade in vivo, in a controlled manner, and ideally at a rate that closely matches the one of formation of the new tissue in the implantation site (Bose et al., 2012; O'Brien, 2011; Rodrigues et al., 2013).

11.3.3 MECHANICAL PROPERTIES

One of the main challenges in bone tissue engineering is, still today, to achieve an adequate balance between mechanical properties and structural requirements, such as high porosity. The bone substitute should be able to support mechanical stresses in the implantation site from the moment of grafting throughout the whole remodeling process, which leads to a further challenge considering that healing times vary with age; tissue of a younger individual normally requires less time to heal than that of the elderly. In addition, the graft should be able to resist surgical handling during the implantation process (O'Brien, 2011).

11.3.4 STRUCTURAL REQUIREMENTS

The structural features of the bone substitute are critical since they can affect both its mechanical properties and its capacity to promote *osteogenesis*, the formation of bone tissue. Bone grafts can be cortical, cancellous, or a combination of both each one with different mechanical and structural properties, which can be used in different applications depending on the surgical requirements. Cortical bone grafts are less frequently used and they are often applied onlay, that is, the graft is applied or laid on the surface of the tissue, to provide structural support and strength. Cancellous bone grafts, on the other hand, lack mechanical strength but are characterized for inducing osteogenesis. This type of bone substitute is commonly implanted in nonunion fractures, maxillofacial and dental defects, spinal fusion, and other small defects (Oryan et al., 2014). Typically, cancellous bone grafts take the form of 3D-porous scaffolds, and the ability to promote adequate osteogenesis greatly depends on its architecture. Scaffolds should be highly porous, to guarantee cellular penetration, and their pores should be interconnected, both to allow the diffusion of nutrients to cells as well as the exit of waste and degradation products out of the scaffold (O'Brien, 2011).

11.3.5 MANUFACTURING TECHNOLOGY

Last but not least, clinical and commercial viability are key factors to consider when engineering bone grafts. The manufacturing process should be cost effective

and easily scalable from laboratorial to industrial production. Additionally, it is important to consider the clinician's point of view, since they are the ones ultimately using the product. For example, they typically prefer a bone graft that can be easily adapted into different shapes and an off-the shelf product ready to be implanted; the fewer extra surgical procedures prior to implantation, the better (O'Brien, 2011).

11.4 OVERVIEW OF COMMERCIALLY AVAILABLE BONE GRAFTS

Solutions for bone repair include the use of materials that, if not autologous, are either of natural or synthetic origin. Biological or natural bone grafts are those obtained from either human or animal origin; thus, autogenous, allogenous, and xenogenous grafts are all within this classification (Kolk et al., 2012). Synthetic grafts, also known as alloplastic grafts, include the use of polymers but mainly ceramic-based materials such as HAp, calcium sulfate, and bioactive glass (Kolk et al., 2012; Kumar et al., 2013). There is also the alternative of using composite and hybrid materials, commonly consisting in the combination of ceramic-based materials with polymers.

Autogenous grafts are obtained from nonessential bones of the same individual receiving the graft. They fulfill all primordial properties for bone regeneration as they are osteoconductive, osteoinductive, and they carry osteogenic cells and growth factors without the risk of transmitting immune or infective diseases. Nevertheless, due to the requirement of a surgical donor site, this type of graft can lead to postoperative pain, blood loss or hematomas, possibility of infection and esthetic deformity, among other complications (Campana et al., 2014).

Bone grafts obtained from another individual of the same species are called allogenic; they are harvested from cadavers, making them readily available in different shapes and sizes. Allografts are also osteoinductive and osteoconductive but not osteogenic, since they lack viable cells. They can be acquired from bone banks unprocessed and frozen, thus including all growth factors and normal ultrastructure. Otherwise, their processing includes steps such as defatting, removing the bone marrow, and commonly freeze-drying, which changes the graft's mechanical properties, making them less resistant. Finally, they have to be sterilized before use. This type of graft overcomes the limitations presented by autografts, but presents some limitations such as high cost, risk of rejection and, additionally, concerns related to disease transmission in spite of undergoing sterilization (Zimmermann and Moghaddam, 2011).

The term *xenograft* refers to bone substitutes acquired from a species other than human, frequently bovine bone tissue. Usually, all organic material is

removed from the animal tissue by a high temperature treatment, followed by a chemical procedure with a strong base, such as NaOH, to obtain a porous HAp material. Xenogenic materials are readily available and show adequate absorption characteristics; however, they exhibit the same disadvantages as allografts, with additional risk of transmission of zoonoses, allergenic response, and a more likely rejection of the graft (Oryan et al., 2014). In addition to ceramic-based xenografts, another readily available biological graft is the so-called demineralized bone matrix (DBM). It is obtained mainly from human, bovine, or equine bone tissue through decalcification and sterilization, resulting in a sponge-like collagen material (Kolk et al., 2012; Zimmermann and Moghaddam, 2011). Some commercially natural bone substitute materials are listed in Table 11.2.

With regard to synthetic materials, ceramic-based grafts are the most commonly used, especially based on HAp and β-tricalcium phosphate (β-TCP), due to the advantages they present in what concerns osteoconductivity, long shelf life, unlimited supply, and absence of risk of disease or virus infection. In addition, they can be produced with different shapes, porosity and composition; commercially they are usually sold as granules, injectable pastes, and cements, among other forms (Zimmermann and Moghaddam, 2011; Dorozhkin, 2010). As for the disadvantages of these alloplastic grafts, the synthesis of HAp and β-TCP with the desire characteristics (Ca/P ratio, crystallinity and morphology, etc.) can be challenging and, despite its hardness and porosity, they are brittle, limiting their applications (Zimmermann and Moghaddam, 2011).

In efforts to improve the overall properties of ceramic materials, they have been combined with other components, mainly polymers, to produce composite and hybrid materials. Many scientists use these terms interchangeably and there is still no clear borderline between them. For the purposes of this work, the terms are used according to IUPAC definitions (McNaught and McNaught, 2014); therefore, a composite is a material constituted by two or more components comprising multiple, different nongaseous phases such that at least one of which is a continuous phase. The term *hybrid* refers to a material composed by an intimate mixture of organic or inorganic components, or both in which normally the components interpenetrate at scales less than 1 μm.

A number of biodegradable polymers, both synthetic and natural, have been used in combination with ceramic materials, including PLA, PGA, PLGA, collagen, alginate, and CS, among others. In this context, the combination with collagen has been widely studied and currently the market offers a variety of ceramic/collagen products. CS-based products, on the other hand, besides the many studies proving its suitability for tissue regeneration, have not yet reached the market at great scale. A list of some commercially available calcium phosphate–based alloplastic composites and hybrid grafts is shown in Table 11.3.

Table 11.2 List of Some Commercially Available Biological Grafts

Company	Product Name	Composition/Source	Form	References
Ace Surgical Supply Co., Inc.	Nu-Oss	Both mineral phase and DBM derived from bovine tissue	Granules and blocks	Ace Surgical Supply Co., Inc. (2016)
	AlloOss	Both mineral phase and DBM derived from human tissue	Particles, blocks and putty	
AlloSource	AlloFuse	Derived from DBM of human tissue	Gel, paste, and putty	AlloSource TM (2016)
	AlloFuse Plus	Derived from DBM and ground cancellous bone of human tissue	Paste and putty	
Biomet*3i*	Endobon	Derived from mineral phase of bovine bone	Granules	Biomet3i TM (2016)
Botiss Dental GmbH	Cerabone	Derived from mineral phase of bovine bone	Granules and blocks	Botiss Dental GmbH (2016)
	Maxgraft	Derived from cancellous and cortical bone of human tissue	Granules and blocks	
D-bone	D-bone	Derived from mineral phase of bovine bone	Granules	D-bone (2016)
DePuy Synthes	Allograft Bone Chips and Blocks	Derived from cancellous and cortical bone of human tissue	Chips and blocks	DePuy Synthes TM (2016)
	DBX Material	Derived from DBM of human tissue in sodium hyaluronate carrier	Putty and strip	
Exactech	Optecure	Derived from DBM of human tissue and a hydrogel carrier provided separately	Dry mix kit (powder with hydrogel)	Exactech (2016)
	Optecure + ccc	Cortical cancellous chips derived from human tissue	Chips (1–3 mm)	
Geistlich Pharma	Geistlich Bio-Oss	Derived from mineral phase of bovine bone	Granules	Geistlich Pharma (2016)
	Geistlich Bio-Oss Collagen	90% Geistlich Bio-Oss (granules) and 10% porcine collagen	Scaffold	
Ost-Developpement	Laddec	Derived from mineral phase of bovine bone	Chips and blocks	Ost-Developpement (2016)
	Lubboc	Derived from mineral phase of bovine bone	Chips, grafts and blocks	

(*Continued*)

Table 11.2 List of Some Commercially Available Biological Grafts *Continued*

Company	Product Name	Composition/Source	Form	References
Osteohealth	Equimatrix	Derived from mineral phase of equine bone	Granules	Osteohealth (2016)
	OSSIF-i sem	Both mineral phase and DBM derived from human tissue	Particles, sponge strip and filler	
	Optimatrix	Derived from DBM of porcine tissue	Membrane	
SigmaGraft Biomaterials	InterOss	Derived from mineral phase of bovine bone	Granules	SigmaGraft Biomaterials (2016)
Tecnoss	Tecnoss mp3 and Tecnoss putty	Derived from mineral phase of equine bone and additional collagen gel	Prehydrated bone mix, putty	Tecnoss (2016)
	Tecnoss Gen-Oss and Tecnoss Chips	Derived from mineral phase of equine bone	Granules and chips	

Table 11.3 List of Some Commercially Available Alloplastic and Composites Grafts

Company	Product Name	Composition	Form	References
Artoss GmbH	NanoBone	HAp (76% for granules, 61% for blocks and putty) and silicon dioxide (24% granules and 39% blocks and putty)	Granules, blocks, and putty	Artoss GmbH (2016)
Baxter	Actifuse	Silicate substituted calcium phosphate	Granules and scaffolds	Baxter (2016)
Berkeley Advanced Biomaterials Inc.	Bi-Ostetic	Tricalcium phosphate and HAp	Granules, blocks, and cylinders	Berkeley Advanced Biomaterials Inc. (2016)
	Bi-Ostetic Foam	Type I bovine collagen, tricalcium phosphate and HAp	Foam	
Biocomposites Ltd.	geneX	β-TCP and calcium sulfate	Putty	Biocomposites Ltd. (2016)
	Allogran-R	β-TCP	Granules	
Ceramed	NeoBone	75% HAp and 25% TCP	Granules, blocks, and wedges	Ceramed (2016)
	NeoCement	Solid phase: tetracalcium phosphate and TCP. Liquid phase: citric acid, CS and apyrogenic water.	Cement (to be mixed before use)	
	k-IBS	Calcium phosphate granules and CS	Injectable gel	
	n-IBS	100% HAp nanoparticles	Injectable	
Curasan	Cerasorb M	>99% β-TCP	Granules	Curasan (2016)
Sunstar Degradable Solutions AG	Guidor *easy-graft*	β-TCP granules coated with PLGA, mixed with BioLinker (*N*-methyl-2-pyrrolidone liquid activator)	Granules	Sunstar Degradable Solutions AG (2016)
DePuy Synthes	chronOS	100% β-TCP	Granules and scaffolds	DePuy Synthes TM (2016)
	Norian	Carbonated apatite and polylactide/glycolide copolymer fibers	Injectable and fast-set putty	
Exactech	OpteMx	60% HAp and 40% β-TCP	Granules and scaffolds	Exactech (2016)

(*Continued*)

Table 11.3 List of Some Commercially Available Alloplastic and Composites Grafts *Continued*

Company	Product Name	Composition	Form	References
Fluidinova	nanoXIM-HAp100	100% HAp nanoparticles in pure water at concentrations 15.0 and 30.0 wt.%	Paste	Fluidinova (2016)
	nanoXIM-HAp200	100% HAp nanoparticles agglomerates	Powder	
	nanoXIM-TCP200	100% Ca-deficient HAp nanoparticles agglomerates with 90% β-TCP phase purity	Powder	
Heraeus Kulzer GmbH	Ostim	100% HAp nanoparticles suspended aqueous phase at concentrations 35.0 wt.%	Paste	Heraeus Kulzer GmbH (2016)
Hoya Technosurgical Corporation	Apaceram	HAp	Particles and scaffolds	Hoya Technosurgical Corporation (2016)
Implandent Ltd.	Osteogen	Calcium apatite (nonspecified)	Powder	Implandent Ltd. (2016)
Kerr	Bioplant	Calcium hydroxide and biodegradable polymer (nonspecified)	Spherical beads	Kerr TM (2016)
Stryker GmbH & Co.	HydroSet Injectable	*Powder:* Di-, tri-, and tetracalcium phosphate. *Liquid:* Sodium phosphate, polyvinyl-pyrrolidone and water	Paste (to be mixed before use)	Stryker GmbH & Co. (2016)
	BoneSave	80% TCP and 20% HAp	Granules	
	BoneSource	HAp	Cement	
	Vitoss BBTrauma	β-TCP and bioactive glass (nonspecified)	Scaffold	
Teknimed S.A.S.	Cementek	Calcium apatite (nonspecified) powder plus separate aqueous solution	Injectable (to be mixed before use)	Teknimed S.A.S. (2016)
Zimmer Biomet	Calcitite	100% HAp	Granules	Zimmer Biomet TM (2016)
	IngeniOs	100% HAp or 100% β-TCP	Particles	

11.5 HYDROXYAPATITE AS A BIOMATERIAL FOR BONE REGENERATION

HAp is a member of a large isomorphous series of minerals called *apatites*, which means "deceivers" in Greek, a name given due to their diversity of form and color. Apatites can be found in nature mainly in mineral deposits and in mammalians bones and teeth (Park, 2009). The apatite profusely found in sedimentary rocks is essentially carbonate fluoroapatite, whereas the one found in bones and teeth is very similar, in terms of crystallography and chemical composition, to HAp (Park, 2009; Kantharia et al., 2014).

The stoichiometry of HAp is represented by the formula $Ca_{10}(PO_4)_6(OH)_2$; it is a double salt of TCP and calcium hydroxide with a Ca/P ratio of 1.67. Deviations from Ca/P stoichiometric ratio can lead to phase impurities in HAp, such as TCP or CaO, that may affect biological responses (Mucalo, 2015). At physiological temperature and pH ranging from 4 to 12, it is considered the most stable and less soluble calcium phosphate of all (Mucalo, 2015; Koutsopoulos, 2001). However, the solubility of HAp, which is partly responsible for vital properties such as biocompatibility and bioactivity, depends on several factors; thus, the study of its dissolution mechanisms and rate is a topic of great interest.

The solubility rate of HAp depends on shape, crystal size, porosity, crystallinity, and crystallite size. In general, it is soluble in acidic solutions, insoluble in alkaline ones and slightly soluble in distilled water, which increases with the addition of electrolytes. Therefore, solubility varies depending on the surrounding environment; for example, in physiological environments it is affected by the presence of amino acids, proteins, enzymes, and other organic compounds.

Barbucci (2002) explains the solubility equilibrium dynamics of calcium phosphates in wet and biological environments, starting from the influence of their chemical nature on the solubility product value. Phosphate-containing compounds in solution are ruled by the following equilibria:

$$H_3PO_4 \leftrightarrow H_2PO_4^- + H^+, \quad pK_a = 2.1192 \tag{11.1}$$

$$H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+, \quad pK_a = 7.2076 \tag{11.2}$$

$$HPO_4^{2-} \leftrightarrow PO_4^{3-} + H^+, \quad pK_a = 12.0000 \tag{11.3}$$

These equations show that in a very acidic solution, $H_2PO_4^-$ species are predominant, near neutrality HPO_4^{2-} is favored, and PO_4^{3-} species are dominant in basic solutions. Consequently, in the presence of Ca^{2+} the precipitation of HAp is favored in a basic environment; thus, with the contribution of the OH^- groups, its solubility product (K_{SP}) is:

$$K_{SP} = \left[Ca^{2+}\right]^5 \left[PO_4^{3-}\right]^3 [OH^-] \tag{11.4}$$

Then, in acidic conditions, below pH values of 4.3−4.8, HAp becomes very slowly soluble and undergoes the following transformation as a result of the decrease in OH^- concentration and the presence of H^+:

$$Ca_5(PO_4)_3OH + 4H^+ \rightarrow 3CaHPO_4 + 2Ca^{2+} + H_2O \quad (11.5)$$

Chemical composition and crystallinity of HAp are two of the main factors directly influencing biological responses. HAp can be found in two different crystal forms: hexagonal and monoclinic. The hexagonal structure is most frequently found, with $P6_3/m$ space group and lattice parameters of $a = b = 9.432$, $c = 6.881$, and $\gamma = 120$ degrees. In this structure, tetrahedral PO_4 groups are held together by interspersed Ca ions. The Ca ions are arranged in two distinct sites, the so-called Ca(I) are accurately aligned in columns, and the Ca(II) are organized in equilateral triangles centered on the screw axis. Finally, the hydroxide groups (OH) are found on the screw axes arranged in a column (see Fig. 11.2) (Ma and Liu, 2009).

The atomic arrangement in HAp structure is directly related with its properties, and thus with its potential biomedical applications. In aqueous media, HAp presents different net charges in its crystal planes; *a* and *b* planes are positively charged, while charges in *c* plane, are negative. Therefore, it is expected that *a* and *b* planes tend to attract acidic groups of proteins, whereas the *c* planes attract basic groups of proteins (Mucalo, 2015; Kandori et al., 2009; Uskoković and Uskoković, 2011). Moreover, morphology of HAp particles has an effect on their

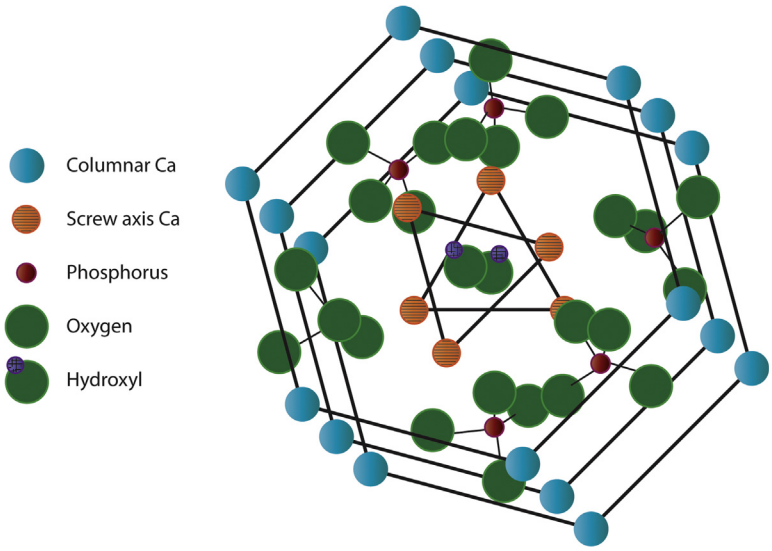

FIGURE 11.2

Schematic representation of hexagonal HAp.

net charge; due to the elongation of HAp particles along the *c*-axis in their typical rod-like morphology, it is believed that the net charge of the particles would be shifted positive, giving a higher specificity of adsorption onto negatively charged proteins (Uskoković and Uskoković, 2011); nevertheless, this is influenced by the pH of the aqueous medium, which, at the same time, influences the dissolution mechanism and rate of HAp, as previously explained.

Dissolution mechanism and rate of HAp are, to a great extent, responsible for HAp's well known osseointegration and bioactivity properties, where *osseointegration* refers to the ability to bind to the bone, whereas *bioactivity* is the ability of a material to induce a specific biological activity such as forming Ca-containing minerals. It has been proposed that these two remarkable properties are due to a mechanism that happens at the HAp−tissue interface after implantation, starting by the dissolution of HAp (Mucalo, 2015; Bertazzo et al., 2010; Ducheyne and Qiu, 1999). Following dissolution, the supersaturated solution at the interface causes the reprecipitation of calcium and phosphate ions, with the formation of carbonate apatite (Ducheyne et al., 1993). At this point, an exchange of ions between the HAp implant and the host bone starts, promoting both bone bonding through the deposition of the ions on the collagenous matrix, and the adsorption of the host bone proteins to HAp surface (Mucalo, 2015; Bertazzo et al., 2010; Ducheyne and Qiu, 1999). Finally, attracted by the high concentration of phosphate and calcium ions, MSCs migrate to the implant surface, differentiate into osteoblasts, and start producing new bone (Ducheyne and Qiu, 1999). An illustration of this mechanism is shown in Fig. 11.3.

Besides chemical composition, crystallinity, and morphology, particle size is another key property that affects biological responses to HAp-containing grafts and, thus, its performance throughout the mechanism described above. In its nanometric form, nanohydroxyapatite has been proven to be more advantageous to the conventional micrometric sized HAp in what concerns the promotion of osteoblast adhesion, differentiation and proliferation, osseointegration, and deposition of Ca-containing minerals on its surface (Supova, 2009; Webster et al., 2000).

Cells are sensitive to the topography of an implant, both at micro- and nanoscale, since it influences factors affecting protein adsorption; among them, surface chemistry, roughness, and surface-free energy (Rouahi et al., 2006; dos Santos et al., 2009). Thanks to their increased surface area and higher percentages of atoms at the surface, nanoscale materials have higher surface energy, wettability, and surface reactivity when compared with conventional sized materials (Gleiter, 1995; Sato and Webster, 2004), which is translated into increased numbers of grain boundaries, to which proteins preferentially adsorb. As a result, grafts containing HAp in its nanometric form have shown enhanced osteoblast adhesion and function, decrease fibroblast adhesion, and enhanced bone remodeling (Tran and Webster, 2009; Yang et al., 2011).

Finally, when it comes to biomedical applications, biodegradation of materials into nontoxic products is highly desirable. In this sense, HAp is an appropriate

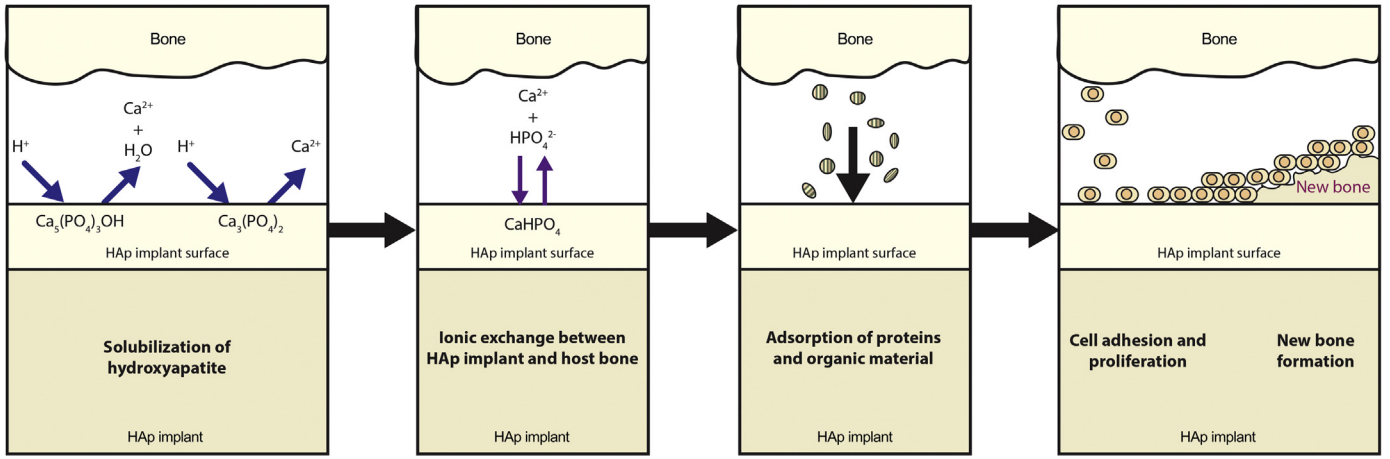

FIGURE 11.3

Schematic diagram representing the proposed mechanism that occurs at the surface of HAp after implantation.

ceramic material, not only for bone regeneration applications but also as a drug delivery system. Its degradation products are Ca^{2+} and PO_4^{3+} ions that already naturally occur in the body, since they are found in the bloodstream at relatively high concentrations (1−5 mM) (Bose and Tarafder, 2012).

11.6 CHITOSAN AS A BIOMATERIAL FOR BONE REGENERATION

CS is the main derivative of natural polymer chitin, the second most abundant natural polysaccharide on Earth, estimated to be produced almost as much as cellulose, with an annual production assessed to be over 10^5 tons worldwide (Zikakis, 2012; Ravi Kumar, 2000). Chitin is a white, inelastic, hard, nitrogenous polysaccharide found in the exoskeleton and in the internal structure of invertebrates, mainly in crustacean shells but also in some microorganisms, yeast, and fungi (Hamid et al., 2013). The use of chitin-based and chitin-derived biomaterials is growing particularly for applications requiring the biodegradability property.

The high nitrogen content gives unique properties to chitin and CS, making them of commercial interest (Georgieva et al., 2012). CS is obtained from the partial deacetylation of chitin, typically by chemical hydrolysis under strong alkaline conditions or by enzymatic hydrolysis under the action of particular enzymes such as chitin deacetylase (Croisier and Jérôme, 2013). Therefore, CS is characterized mainly by its degree of deacetylation (DD); to be considered CS DD should be at least 50% (Costa-Pinto et al., 2011). Structures of both natural polymers are shown in Fig. 11.4.

CS is a linear polymer of (1→4)-linked 2-amino-2-deoxy-β-D-glucopyranose characterized mainly by a DD usually ranging from 50% to 95%, and molecular weight, which can vary from 50 kDa to more than 1000 kDa depending on its source and preparation method (Costa-Pinto et al., 2011; Di Martino et al., 2005; Domb and Kumar, 2011). It is a semicrystalline compound, with crystallinity dependent on its DD, and soluble in aqueous acidic solutions, where its solubility is mostly dependent on the protonation of amino groups (Costa-Pinto et al., 2011), which facilitates its processability, in opposition to some currently used materials in biomedical applications (Ravi Kumar, 2000).

CS presents a wide list of desirable properties for bone regeneration and controlled drug delivery, namely biocompatibility (Bavariya et al., 2014; Shin et al., 2005); mucoadhesiveness (He et al., 1998; Lehr et al., 1992); hydrophilic character, which promotes osteoblast adhesion and proliferation (Seol et al., 2004); wound healing properties (Azad et al., 2004; Ueno et al., 1999); and nontoxic biodegradation products (Croisier and Jérôme, 2013; Di Martino et al., 2005). Most of these properties are caused mainly by its cationic nature. This polymer contains

in its main backbone primary amino groups that become positively charged in acidic medium, as shown in Fig. 11.5 (Samal et al., 2012).

With a pK_a around 6.0–6.5 (Samal et al., 2012; Dash et al., 2011), it is both reactive and soluble as a function of pH, presenting solubility in the majority of organic acidic solutions including formic, acetic, tartaric, and citric acid

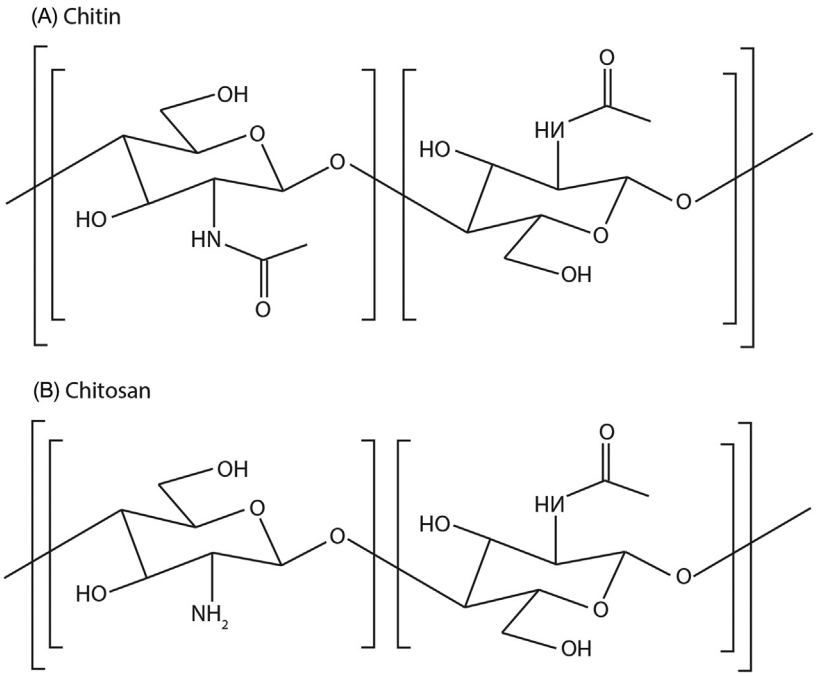

FIGURE 11.4

Structure of (A) chitin and (B) CS.

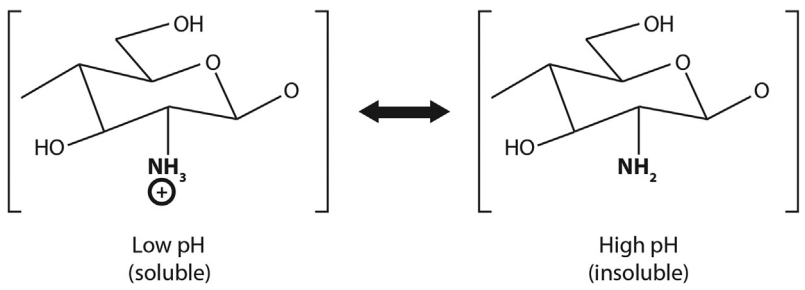

FIGURE 11.5

Cationic nature of CS.

(Nagpal et al., 2010). Thus, at low pH, the amino groups are protonated and render CS a cationic polyelectrolyte. This polyelectrolyte property has been employed to generate multilayer films or capsules using layer-by-layer assembly (Berth et al., 2002; Picart et al., 2005). Otherwise, at high pH the amines are deprotonated and CS changes from a soluble cationic polyelectrolyte to an insoluble polymer.

A notable effect of CS's polyelectrolyte behavior is the improved adhesion and proliferation of cells in the surface of CS-containing materials, caused by the electrostatic interaction of the positively charged CS with the negatively charged glycosaminoglycans (GAG), proteoglycans, and other negatively charged molecules. GAG and proteoglycans are noncollagenous ECM proteins that regulate and direct the construction and maintenance of the ECM (Burr and Allen, 2013). Given that both cytokines and growth factors bond to GAG, the formation of CS-GAG complexes in a bone graft is beneficial because they can promote the retention and concentration of these desirable substances on its surface (Di Martino et al., 2005; Kim et al., 2008).

In swollen state, CS acts as a natural bioadhesive, an ability that has been of special interest for drug delivery applications (Costa-Pinto et al., 2011; Şenel and McClure, 2004; Bertram and Bodmeier, 2006). The mucoadhesive property of CS is caused by its electrostatic interaction between positively charged CS and negatively charged residues, namely sialic acid, on mucosal surfaces (Croisier and Jérôme, 2013). Another feature of CS attributed to electrostatic interactions is its capacity to induce hemostasis, which has been related to interactions of positive charges of CS with negatively charged red blood cell membranes (Croisier and Jérôme, 2013; Rao and Sharma, 1997; Ong et al., 2008).

The antimicrobial activity of CS, a fundamental property in wound healing processes, has been widely studied (Ong et al., 2008; Altiok et al., 2010; Saravanan et al., 2011; Shi et al., 2006). In what regards orthopedic implants, bacterial infection is a major concern due to the severe consequences it can lead to, such as implant failure, hospitalization, and sometimes even mortality of the patient (Saravanan et al., 2011). Mechanisms explaining antibacterial and antifungal activity of CS are more complex. The first mechanism proposes that the high affinity of cationic groups of CS toward the anionic components in microorganisms' cell wall, such as Gram-negative lipopolysaccharide and cell surface proteins, alters the cells' permeability preventing the mass transport of essential materials across the cell wall. The second mechanism proposes the inhibition of the microbial RNA synthesis due to the bonding of protonated amino groups of CS with the cell DNA (Croisier and Jérôme, 2013; Domb and Kumar, 2011; Kong et al., 2010).

Another important property of CS is its biodegradability. A wide range of biomedical applications, including matrices for controlled drug release, resorbable devices such as bone cements, and scaffolds for tissue engineering, require the controlled degradation of the biomaterials over time to let the natural

tissue develop and completely replace the foreign element (Reis et al., 2008). CS's biodegradability takes place by lysozyme action, an abundant enzyme found in the human body, through the degradation into small glycomino chains, with degradation rate being inversely related to the DD. Regarding the biocompatibility of the degradation products, it has been reported that these products undergo a quick elimination by the kidney, thus avoiding accumulation in the body (Costa-Pinto et al., 2011; Reis et al., 2008).

In addition to all the above-mentioned desirable properties, including the solubility of CS in aqueous acidic medium that allows its processing under mild conditions (Levengood and Zhang, 2014; Croisier and Jérôme, 2013) together with CS's ability to be shaped into different forms such as porous scaffolds (Araujo et al., 2014; Ji et al., 2011; Siddiqui et al., 2015), fibers (Aklog et al., 2015; Albanna et al., 2013), sponges (Seol et al., 2004), and microparticles (Custódio et al., 2015; Obaidat et al., 2015; Shen et al., 2014), are advantageous features for tissue engineering applications (Costa-Pinto et al., 2011). Another advantage is its availability in large quantities and at adequate commercial grades (Bansal et al., 2011).

11.7 HYDROXYAPATITE/CHITOSAN NANOCOMPOSITE MATERIALS

The combination of n-HAp with CS has been of special interest for biomedical applications, particularly for bone regeneration. HAp by itself is highly brittle, whereas CS, despite its capacity to promote osteogenic cells' attachment and proliferation, it does not induce the deposition of bone minerals on its own. Therefore, when combining both components, HAp provides the required bioactivity while CS adds elasticity to the final material, resulting in a great candidate for nonload-bearing bone graft applications (Levengood and Zhang, 2014; Dorozhkin, 2011). However, even though combining HAp and CS can be advantageous, the production of such hybrid system still faces several challenges.

The final properties of any hybrid inorganic–organic system are affected by several important aspects that must be considered. A hybrid material consists of at least two phases separated by an interface, in which one of the phases is usually inorganic, consisting of filler particles, dispersed in the second phase, a polymeric matrix. The filler's shape and size, properties and volume percentage, the matrix properties such as molecular weight, the dispersion stability of the filler in the polymeric matrix, and the interactions in the filler/matrix interface are some of the main factors influencing the properties of the hybrid (Supova, 2009). Furthermore, in the case of hybrid materials for biomedical applications, biocompatibility, degradation rate, and nontoxicity must be considered as previously discussed.

From a chemical point of view, there are several ways to incorporate inorganic particles (fillers) into organic polymers, depending on the interactions between the constituents, either strong, weak, or without any chemical interactions between them. Strong interactions can be covalent, coordination type, and ionic bonds, whereas van der Waals, hydrogen bonds, and hydrophilic−hydrophobic are considered weak interactions. Inorganic−organic hybrid materials can be classified based on such interactions and the different arrangements of the polymer chains relative to the inorganic nanoparticles. In this regard, Kickelbick (2003) proposed four different types of arrangements, as shown in Fig. 11.6.

The first arrangement (Fig. 11.6A) takes place when, in the absence of strong interactions, the inorganic particles are dispersed in the organic matrix such that, depending on the functionalities of the components, the inorganic units undergo weak cross-linking through physical interactions with the polymer or, the

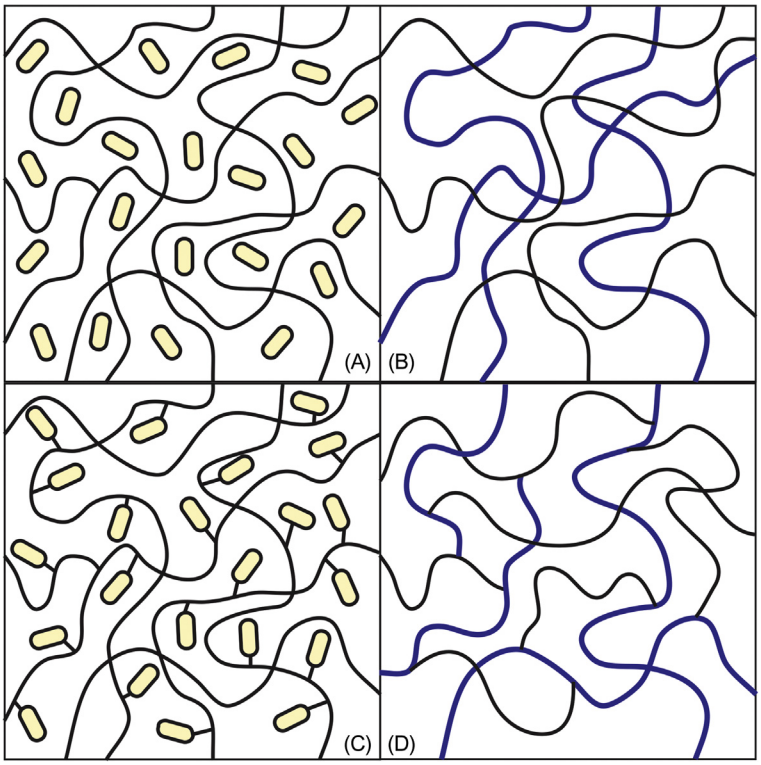

FIGURE 11.6

Schematic representation of different types of inorganic−organic hybrid materials based on polymeric chain arrangements relative to the inorganic particles: (A) inorganic particles embedded into the organic polymer; (B) IPNs; (C) inorganic particles chemically bound to the polymer backbone; and (D) dual inorganic−organic hybrid polymer.

inorganic particles are entrapped in a cross-linked polymeric matrix (Kickelbick, 2007). Interpenetrating polymer networks (IPNs) consist of a polymer comprising two or more networks that are, on a molecular scale, partially or totally interlaced but not covalently bound (Fig. 11.6B). In the case of inorganic−organic IPNs, formed for example by sol−gel reactions, one of the networks is formed by the inorganic component, and they can be processed either simultaneously or sequentially (Alemán et al., 2007). Finally, the hybrids can be produced by discrete (Fig. 11.6C) or interconnected (Fig. 11.6D) inorganic particles attached to the polymer backbone by covalent bonds (Kickelbick, 2003, 2007).

The chemical nature of CS makes it a versatile polymer as it can be either chemically or physically cross-linked. Chemical cross-linking is irreversible since a polymeric network is formed by covalent bonds, requiring the assistance of a cross-linking agent such as glutaraldehyde or genipin. Physical cross-linking, on the other hand, is reversible since linkage occurs through van der Waals forces, ionic attractions, hydrogen bonding, or hydrophobic interactions (Reis et al., 2008; Berger et al., 2004). Considering this, the incorporation of HAp nanoparticles into a CS matrix can be done following several techniques as described in the next section.

With advances in technology, various methods for producing HAp/CS nanocomposite materials have been reported, following both chemical and physical paths. In general, the production of HAp/CS materials requires a first stage of dispersion preparation, in which HAp and CS are combined to produce the base material that, in a second stage, is shaped into the final form. Thus, the second stage consists of the fixation of the structure—into microparticles, granules, scaffolds, cements, etc.—carried out by inducing phase separation and the elimination of the solvent. Afterward, the produced nanocomposites usually have to be subjected to purification and sterilization processes, since their potential applications are within the biomedical field. One of the key issues in these sequential procedures is to guarantee that none of the stages compromises, among other desirable properties, the morphological and architectural features of the final envisaged forms (scaffolds, powders, etc.) to ensure a suitable environment for cell attachment and proliferation. Fig. 11.7 shows schematically the stages used for the HAp/CS nanocomposite materials.

The dispersion preparation is a key stage of the productive process. Here the HAp is incorporated into the CS matrix. This step can be done in a number of ways; among all the methods reported, in situ coprecipitation and simple mixing are the most popular ones (Levengood and Zhang, 2014; Venkatesan and Kim, 2014).

In the so-called in situ coprecipitation the synthesis of HAp is carried out in the presence of CS in neutral or basic environment ($pH \geq 7$), therefore, causing the precipitation of CS along with HAp simultaneously (Yamaguchi et al., 2001; Chen et al., 2002; Danilchenko et al., 2011). Yamaguchi et al. (2001) developed a methodology that consisted of dripping CS in H_3PO_4 solution on a calcium hydroxide suspension. Homogeneous incorporation of HAp into the CS matrix has been reported using this process, however, the fact that the formation of HAp

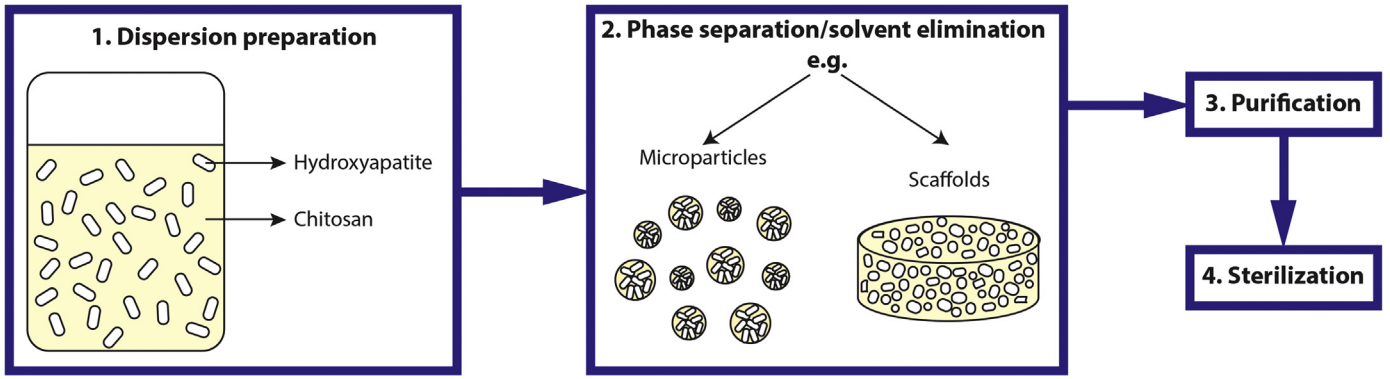

FIGURE 11.7

Schematic representation of the stages used for the HAp/CS nanocomposite materials.

occurs in the presence of CS can hinder the synthesis of high purity HAp and the achievement of the desired morphology.

An alternative method consists of physically mixing previously synthesized HAp particles, usually in powder form or ultrasonically dispersed in water, into a CS solution. Typically, this technique, also called *simple mixing* method, is carried out at lab-scale, by mixing both components in a vessel using a magnetic stirrer, resulting in final nanocomposite materials with quite favorable physicochemical and biological properties (Kim et al., 2009; Thein-Han and Misra, 2009; Zhao et al., 2002). This is a simple and straightforward technique that can also be advantageous in terms of reproducibility relative to the coprecipitation method; nevertheless it can lead to nonhomogeneous dispersions at a microscopic level due to the difficulty in controlling the mixing between the two dissimilar phases that can undergo phase separation (Supova, 2009; Peniche et al., 2010) and, therefore, originate final materials with weaker mechanical properties when compared with other methods (Hu et al., 2004; Chen et al., 2011).

An important parameter to consider when preparing n-HAp/CS hybrid dispersions is the pH, especially if HAp has to be introduced into an acidic environment that can influence its solubility, chemistry, and phase stability (Ito et al., 1996). When the method of physically mixing both components is used, HAp is often introduced in an acidic CS solution, resulting in dispersions with pH typically around 4, potentially causing the dissolution of HAp (Wilson and Hull, 2008). To the best of our knowledge, Wilson and Hull (2008) is the only report where HAp surface chemistry was studied after aging nanophase HAp particles in 0−2.5 wt.% CS acetate solutions for 30 days. After incorporation of HAp into CS solution, the HAp/CS mixtures had a pH around 4−5. The authors reported changes in HAp surface chemistry, colloid stability, and chemical composition after long-term aging of HAp in CS acetate gel solutions. Such changes were attributed to solubility effects, since HAp becomes slowly soluble at pH values lower than 5. It was also found that CS strongly adsorbs to HAp particles and improves colloid stability.

Following the dispersion preparation, the separation/solvent elimination stage involves the structural fixation of the n-HAp/CS nanocomposite material in its final form (e.g., microparticles, granules, pastes, scaffolds, cements, etc.), for which a number of methods can be used. The main techniques used for the production of microparticles and scaffolds, two of the most attractive structural forms of n-HAp/CS nanocomposites, are described in the next sections.

11.8 PREPARATION OF HYDROXYAPATITE/CHITOSAN MICROPARTICLES

The combination of HAp with CS for bone tissue engineering applications is typically directed to the production of porous scaffolds; nonetheless, advances in

science and technology have led to the development of particulate materials. The use of microparticles in tissue engineering and regenerative medicine can be very beneficial from a functional point of view; these are very versatile structures that, thanks to their reduced size, can be used as injectable systems, shaped into a solid substrate with increased surface area capable to promote chemical and biological reactions, or even be embedded into nanocomposite scaffolds for controlled release of bioactive substances (Oliveira and Mano, 2011; Silva et al., 2007a,b). In this context, the development of a particulate system containing n-HAp in a micrometer-sized matrix is advantageous; this approach allows HAp's superior properties at the nanoscale to be preserved in the form of microparticles that are easier to handle (Okuyama et al., 2006).

Emulsification is one of the most common methods used in the pharmaceutical industry for the production of microparticles. This method is based on the formation of an emulsion consisting of the n-HAp/CS dispersed into a nonmiscible phase. The presence of CS, which acts as a hydrogel precursor, allows the formation of the microparticles by hardening according to its sol−gel mechanism (Gasperini et al., 2014). Ding et al. (2012) produced HAp/CS composite microspheres by a water-in-oil (W/O) emulsion method such that HAp was precipitated in situ. For that, $Ca(NO_3)_2$ was added to a CS solution and the mixture was emulsified in vegetable oil. Afterward, a phosphate source (Na_2HPO_4) was added to the emulsion, followed by the addition of cross-linking agent glutaraldehyde and NaOH to induce the precipitation of the HAp within the CS matrix. As final product, the authors obtained 10-μm HAp/CS composite microspheres with n-HAp particles homogeneously distributed in the CS. In addition, authors reported that the produced n-HAp was poorly crystalline and contained carbonate ions. Li et al. (2010) also produced HAp/CS composite particles by emulsification. In this case, the authors used a multiple water-oil-water emulsion method in which a mixture of $Ca(NO_3)_2{\cdot}4H_2O$, CS and $(NH_4)_2HPO_4$ solutions were used as outer and inner aqueous phases respectively. Thus, the precipitation of HAp was carried out in situ and glutaraldehyde was used as cross-linking agent to finally produce the particles. Authors reported the production of core−shell HAp/CS composite nanospheres with 100- to 200-nm diameter.

Another appealing method for the production of n-HAp/CS microparticles is by spray drying. Among the existing techniques, spray drying is a promising technology for the manufacturing of microparticles with controllable size and morphology (Nandiyanto and Okuyama, 2011). It is a process widely used as a microencapsulation/stabilization technique in the food (Dias et al., 2015; Ribeiro et al., 2015) and pharmaceutical (Sollohub and Cal, 2010; Song et al., 2015) industries. Easy industrialization, cost-effectiveness, and continuous production are attractive features of this technique; however, special care must be taken with heat-sensitive materials, since prolonged contact with high temperatures can cause product degradation (Lima et al., 2012). This method is based on the drying of atomized droplets of a previously prepared solution or emulsion/dispersion using a cocurrent stream of hot gas.

Production of HAp/CS microparticles by spray drying was reported by Başargan et al. (2015) (Burr and Allen, 2013). In the approach used by the authors, a HAp/CS-slurry using the coprecipitation method (pH $\geq$ 9.0) was prepared, followed by spray drying. The effect of inlet temperatures (120°C and 160°C) and different HAp/CS weight ratios was studied. Spherical microparticles of mean size 4−6 μm were produced, and an increase in the surface area with the addition of CS, as well as with the increase of inlet temperature, were observed. However, the FTIR analysis suggested the substitution of carbonate in the apatite structures, compromising the purity of HAp. Thermal degradation studies were not reported.

More recently, Ruphuy et al. (2016) produced n-HAp/CS composite microparticles by spray drying, using a standard nozzle of 0.7 mm of diameter, and inlet temperature of 170°C and feed rate of approximately 4.5 mL/min as operating conditions. The produced n-HAp/CS nanocomposite microparticles contained highly pure, nanometric HAp (50 nm). The work studies the effect of pH and the presence of salts on the final microparticles' size and morphology. For that, n-HAp/CS nanodispersions were firstly prepared by simple mixing method at two different pH values, above (pH 7) and below (pH 5.5) CS pK_a (6.5). The presence of salts was analyzed by preparing the nanocomposites with and without KCl. It was concluded that different types of spray-dried microparticles are produced depending on the pH at which the initial nanodispersions are produced; the microparticles produced at lower pH (pH 5.5) were preferred over the particles produced at higher pH, which required an extra step in the production process and had a high tendency to form large agglomerates. Relative to the presence of KCl, it was determined that it has no beneficial effect on the stability of the precursor nanodispersions and its presence could hinder cells' metabolism by creating a hypertonic environment. In addition, it was proved that CS was not degraded during the spray drying process at the conditions used since no differences were observed when comparing the thermogravimetric (TG) plots of the spray-dried microparticles with TG plots of analogous of freeze-dried samples. Fig. 11.8 shows the SEM images of the obtained microparticles.

Besides W/O emulsion (Ding et al., 2012) and spray drying (Ruphuy et al., 2016; Başargan and Nasün-Saygılı, 2015), other methods that have been exploited to produce n-HAp/CS microparticles are supercritical assisted atomization (Reverchon and Adami, 2013), spray coagulation (Granja et al., 2004), and electro-spray coagulation (Chen et al., 2015). In the supercritical assisted atomization method, a supercritical fluid (commonly supercritical CO_2) acts as a nonsolvent for the composite such that, when the n-HAp/CS composite dispersion is sprayed in the supercritical fluid, CS precipitates (Oliveira and Mano, 2011). Coagulation processes are based on polyelectrolytes ability to cross-link and form hydrogels; thus, in spray coagulation, the n-HAp/CS composite dispersion is sprayed into a bath solution under stirring, which contains a solution that promotes the hardening/gelation of CS (usually NaOH solution) (Dias et al., 2015; Lima et al., 2012). Lastly, electro-spraying consists of applying an electric

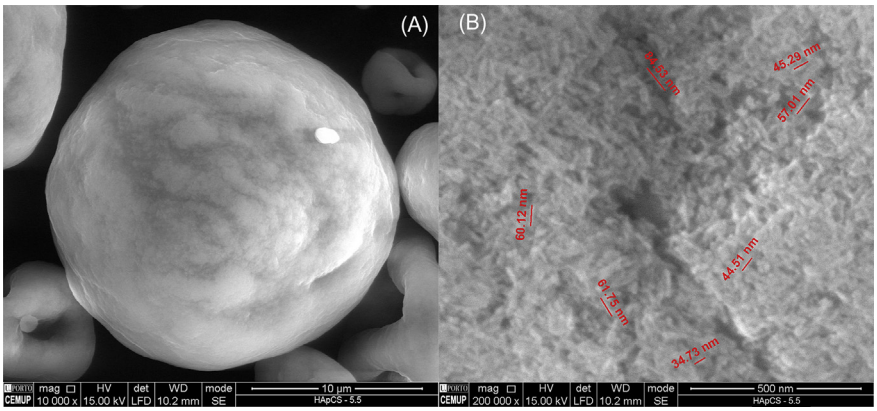

FIGURE 11.8

SEM images of the microparticles obtained by spray drying at lower pH (pH 5.5): (A) 10,000× magnification showing one whole microparticle and (B) 200,000× magnification showing the HAp nanoparticles at microparticle surface.

field to the composite dispersion extruded from a syringe. The high voltage potential applied makes the dispersion form a jet that, under specific parameters, allows the formation of the microparticles (Oliveira and Mano, 2011).

11.9 PREPARATION OF HYDROXYAPATITE/CHITOSAN SCAFFOLDS

The most typical structure produced for bone regeneration is *scaffold*. Taking advantage of the remarkable ability of bone self-regeneration, the idea behind a scaffold is to act as 3D template that once implanted in the bone defect, allows cell migration, adhesion, proliferation and eventually, the formation of new bone tissue. Considering this, microstructure is a key factor to be evaluated when producing scaffolds for bone regeneration. A combination of adequate pore sizes and interconnectivity is needed to allow the motion of cells throughout the scaffolds, as well as of the required nutrients and metabolites for cells' survival and proliferation (O'Brien, 2011). Several studies have been conducted regarding the relationship between pore size and cell activity, showing that both pores $>100\,\mu m$ and pores $<50\,\mu m$, are important (Bose et al., 2012). Macropores ($>100\,\mu m$) allow angiogenesis and contribute to nutrient and waste transport, whereas pores $<50\,\mu m$ can improve osteointegration and generate cell anchoring sites (Agrawal and Ray, 2001; Lan Levengood et al., 2010; Polak et al., 2011; Woodard et al., 2007; Klenke et al., 2008). Despite the advances achieved today, the production of the ideal bone scaffold still remains a challenge, mainly in what concerns the accomplishing of a balance between microstructure (high porosity with ideal pore

sizes) and mechanical properties (sufficient compression strength for a particular application).

The most frequently used technique to prepare n-HAp/CS scaffolds is freeze-drying. This is a simple procedure in which phase separation is induced by the formation of ice crystals upon freezing, followed by the elimination of the solvent by the sublimation of the ice crystals, producing the final nanocomposite 3D-porous structure (Levengood and Zhang, 2014; Costa-Pinto et al., 2011). Many authors using this technique obtained n-HAp/CS based nanocomposite scaffolds with desirable high porosity and interconnected pores (Thein-Han and Misra, 2009; Zhao et al., 2002; Kong et al., 2005, 2006; Oliveira et al., 2006; Jiang et al., 2008; Sultana et al., 2015). This technique, however, exhibits some limitations. Its major drawback is, probably, the formation of a surface skin caused by the collapse of the porous structure at the scaffold−air interface due to the interfacial tension generated during solvent evaporation (Levengood and Zhang, 2014). To avoid this phenomenon, temperature control must be rigorous such that an adequately low temperature is guaranteed to support the interfacial tension (Costa-Pinto et al., 2011). In addition, mechanical properties of these structures produced by freeze-drying are very limited, even if the scaffold is subjected to cross-linking of the CS (Costa-Pinto et al., 2011).

Zhang et al. (2015) recently studied the influence of the freeze-drying process on the final properties of HAp/CS nanocomposite scaffolds. For that, HAp/CS nanodispersions were prepared by simply mixing HAp nanoparticles in a CS solution. After dispersing the HAp particles by ultrasonication, the mixture was poured in cylindrical molds and subjected to different freezing methods (e.g., rapid cooling method was achieved using liquid nitrogen, whereas slow cooling method was carried out by placing samples in the freezer at $-40^{\circ}C$ for 24 hours). The authors concluded that the prefreezing method has a great influence on the morphology and mechanical properties of the HAp/CS nanocomposite scaffolds. The scaffolds obtained by the slow cooling method exhibited both higher porosity and compression strength, comparatively with the ones obtained using the rapid cooling method.

Other popular phase separation/solvent elimination techniques to produce n-HAp/CS scaffolds are particulate leaching (Oliveira and Mano, 2011; Silva et al., 2007a), gas foaming (Silva et al., 2007b), and freeze-gelation (Okuyama et al., 2006). Particulate leaching is a multistep process, in which a porogen agent, such as salts, sugars, paraffin or gelatin, is mixed with the HAp/CS dispersion; next, the dispersion is subjected to freeze-drying, and finally, the porogen agent is leached out by immersion of the scaffold into a solvent solution, generating a final scaffold with two levels of porosity (one produced from the freeze-drying process and another one due to the porogen agent) (Levengood and Zhang, 2014). In gas foaming the formation of a porous structure is induced by the nucleation and growth of gas bubbles within the nanocomposite matrix by using a chemical foaming agent, which can leave unwanted contaminations, or a subcritical or

supercritical gas, for example, nitrogen or carbon dioxide, as blowing agents (Duarte et al., 2009a). Finally, in freeze-gelation pore formation occurs by freezing the nanocomposite dispersion followed by phase separation due the formation of solvent ice crystals. Then, the frozen scaffold is immersed, under freezing conditions, in a gelation solution bath, usually a mixture of NaOH and ethanol, thus inducing gelation and solvent exchange simultaneously. The final HAp/CS nanocomposite scaffold is obtained by air-drying (Rogina et al., 2015; Mu et al., 2010).

In most cases, the obtained n-HAp/CS nanocomposite material still presents residual substances left from the used process. The residual substances are reagents in excess, namely bases (e.g., NaOH) when the synthesis of HAp is carried out during the coprecipitation method or acids (e.g., acetic acid), which are used to solubilize the CS in the case of the simple mixing method. Thus, a purification/neutralization step is necessary to eliminate these residual substances that can compromise the material properties (cause structure disruption, or even inhibit cell growth and proliferation). The presence, for example, of residual acetic acid can cause scaffold disruption due to CS dissolution in acidic aqueous medium (Costa-Pinto et al., 2011). Additionally, to promote cell survival and proliferation, the culture environment must fulfill some fundamental physiological requirements, both in terms of composition and pH (Davis, 2011), which can be affected by the presence of acid and/or alkaline impurities.

Typically, purification/neutralization of n-HAp/CS scaffolds prepared by freeze-drying from dispersions obtained by the simple mixing method, which contain residual acetic acid remaining from CS solubilization, is carried out by immersion in an alkaline medium, washing with ultrapure water, and drying (usually through another freeze-drying step) (Levengood and Zhang, 2014; Thein-Han and Misra, 2009; Kong et al., 2005, 2006; Jiang et al., 2008; He et al., 2011). This neutralization process to remove the residual acetic acid is time consuming and can lead to low purity materials (i.e., materials with residual salts remaining from the used alkaline solution), as well as to materials with compromised structural integrity; the second freeze-drying step applied to the already formed scaffold can damage the porous structure. In fact, it has been reported that CS-based scaffolds undergo shrinkage and distortion after neutralization with NaOH solutions (Madihally and Matthew, 1999; Seda Tığlı et al., 2007).

11.10 HYDROXYAPATITE/CHITOSAN NANOCOMPOSITE MATERIALS STERILIZATION

As with any medical device that is implanted in the human body, n-HAp/CS nanocomposite materials produced for biomedical applications must be subjected to sterilization. The term *sterilization* refers to a procedure, either chemical or physical, whose purpose is to destroy all microbial life, including highly resistant

bacterial endospores (Rao and Sharma, 1995). Typical sterilization techniques include saturated water steam (autoclave), dry heat, ethylene oxide (EtO), and gamma irradiation. These techniques act either physically or chemically, and have proven to be effective eliminating microorganisms, however, they can induce changes in the macromolecular structure of polymeric materials by chain scission, oxidation, hydrolysis, and depolymerization, depending on the polymer's nature and the used sterilization technique (Juan et al., 2012). Studies conducted on CS-based materials have shown that these common sterilization techniques may cause irreversible modification in CS structure and function (Szymańska and Winnicka, 2015).

Therefore, when designing n-HAp/CS nanocomposite materials, sterilization is an important procedure to consider. Given that CS is a thermosensitive material, common sterilization techniques, such as dry heat and steam, are not appropriate since they can cause thermal degradation of the material. Jarry et al. (2001) studied the effects of steam sterilization on CS-based gels by autoclaving samples at 121°C for different times ranging from 0 to 60 minutes. Results from this study showed the degradation of CS after time periods as short as 10 minutes of autoclaving; the samples exhibited 30% decrease in molecular weight, three- to fivefold reduction in dynamic viscosity, and the mechanical properties of the CS-based gels, at 37°C, were significantly weakened. Likewise, Lim et al. (1999) analyzed the effects of heat treatment (dry heat and saturated steam) on the physical properties of CS. Temperatures from 60°C to 160°C were tested for time periods ranging from 0.5 to 4 hours. The samples experienced a change in color and lower water affinity (decreased aqueous solubility), which was attributed to heat-induced reactions involving the NH_2 groups, the formation of cross-links and chain rearrangements. Authors pointed out the need to further analyze if the colored samples have adverse effects on biocompatibility.

EtO and gamma irradiation, on the other hand, can affect CS chemical properties. The use of EtO risks the deposition of toxic residues on the materials surfaces and high-energy gamma irradiation can cause material degradation as well (Checinska et al., 2011). França et al. (2013) reported chemical changes on CS surface after exposure to EtO sterilization independently of its DD, which were attributed to oxidation of the amine groups. The experiments were conducted by exposing CS, in the form of powder, to EtO sterilization (30% EtO and 70% CO_2) during 8 hours at 40°C and 40%−50% relative humidity. Furthermore, Marreco et al. (2004) reported a decreased in tensile strength of CS membranes after sterilization with EtO under the same conditions (30% EtO and 70% CO_2 for 8 hours at 40°C, and 40%−50% relative humidity). In this study, different sterilization methods were applied to CS membranes and the effects on morphological, mechanical properties, and cytotoxicity analyzed. Relative to gamma irradiation, Lim et al. (1998) conducted studies on CS films by irradiating samples with different doses, up to 25 kGy, at 0.73 kGy/h dose rate. Results showed that molecular weight of CS decreased considerably along with an increase in DD, with dependency on the gamma-irradiation dose.

Additionally, after irradiation exposure, water sorption capacity of the samples also decreased.

In this context, the use of supercritical CO_2 ($scCO_2$) as an innovative sterilization technique has recently emerged (Checinska et al., 2011; Dillow et al., 2000; Hemmer et al., 2007; Kamihira et al., 1987; White et al., 2006; Zani et al., 2013; Zhang et al., 2006a,b) with high potentiality to sterilize n-HAp/CS composite materials. Supercritical CO_2 gathers a large amount of advantages that make it attractive for this particular application; it is an environmentally benign, nontoxic, nonflammable, noncorrosive, readily available, and inexpensive solvent (Duarte et al., 2009b; Quirk et al., 2004). This supercritical fluid has been well known for its outstanding solvent capacities due to its high diffusivity; it has been widely used in the food industry as an extraction medium, but also for processing pharmaceuticals due to its low critical temperature (Duarte et al., 2009a). Ruphuy et al. (2018) produced n-HAp/CS scaffolds using a $scCO_2$ assisted process in which $scCO_2$ was used for both purification and sterilization of the scaffolds in one single step. The overall process consisted of three simple stages: first, the preparation of the n-HAp/CS nanodispersions, followed by the structural fixation of the scaffolds by freeze-drying, and finally, the $scCO_2$ extraction. The latter was carried out in batch mode at a constant pressure of 8.0 MPa, and different extraction parameters were tested, namely temperatures of 40°C and 75°C and the number of cycles. It was determined that, by subjecting the scaffolds to the best-achieved conditions (two cycles at $T = 75°C$ and $P = 8.0$ MPa) it was possible to remove 80% of the residual acetic acid. Furthermore, results from the microbiological assay showed no microbial growth on the scaffolds produced under such conditions (proving that the scaffolds were sterile), and the in vitro tests showed that the scaffolds were cytocompatible and osteoconductive. Finally, even when further studies are needed to validate $scCO_2$ extraction as an sterilization technique, the authors were able to produce scaffolds with desirable interconnected porous structure, fast swelling, and adequate pore sizes, using a process that allows the microstructural preservation of the scaffolds in contradistinction to other conventional methods.

11.11 CONCLUSIONS

HAp is an outstanding material exhibiting highly desirable properties for biomedical applications, mostly for bone regeneration, due to its similarity with the bone mineral phase. It has been combined with several polymers aiming at producing materials with improved properties. Particularly, its combination with CS has been of great interest for the production of nonload-bearing bone grafts, given the possibility to generate materials mimicking bone composition and with improved

properties, namely in what concerns elasticity. In addition, CS provides a wide list of interesting properties: it promotes osteoblasts' adhesion and proliferation, possesses antimicrobial properties, and it can be processed using mild conditions and can be shaped into different forms, to name some.

Commercially available products for bone regeneration still consist, to a great extent, of biological grafts, either from human or animal sources. However, these grafts present extensive drawbacks, namely, high cost, risk of rejection, risk of diseases and zoonoses transmission, allergenic responses, etc. In what concerns synthetic materials, the market offers a variety of calcium phosphate–based products, alone or combined with other components, mainly collagen. In the performed survey, only one company commercializing calcium phosphate–based products containing CS was found, showing that, besides the many studies proving its suitability for tissue regeneration, HAp/CS nanocomposite materials have not yet made the leap from the laboratory to the market.

Relative to the production of n-HAp/CS nanocomposite materials envisaging biomedical applications, a sequence of productive steps must be followed. Generally, the process starts with the dispersion preparation, in which n-HAp is incorporated into the CS solution. The second stage involves a phase separation/solvent elimination process in which the final structural form (e.g., microparticles or scaffolds) is fixed. Finally, as their potential applications are within the biomedical field, the produced nanocomposite materials have to be subjected to purification and sterilization processes.

The preparation of n-HAp/CS nanocomposite dispersions requires the introduction of HAp into an aqueous environment that can influence its solubility, surface chemistry, and phase stability, affecting, simultaneously, the HAp-nanoparticles/CS-matrix interface interactions and, therefore, the material's final properties. The phase separation/solvent elimination stage determines important structural features of the final product, such as pore size and interconnectivity, particle size, and morphology, and can dictate the need for subsequent purification processes. Purification is a fundamental procedure that can be time consuming, ineffective, and change the properties of the obtained n-HAp/CS nanocomposites.

Most common methods available today for sterilization can cause thermal degradation or deposition of toxic residues on the material's surface. In this context, supercritical CO_2 gathers a large amount of advantages, emerging as a technique with high potentiality to sterilize n-HAp/CS composite materials, without causing structural disruption or compromising the physicochemical and mechanical properties of final composite materials.

Finally, the overall process has an important influence in the final product in terms of efficiency, costs, reproducibility, properties, and versatility. It is of utmost importance from a clinical point of view to have ready-to-use products without requiring extra steps prior to use, and materials that can be easily adapted into different shapes.

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