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# Manufacturing methods, properties, and potential applications in bone tissue regeneration of hydroxyapatite-chitosan biocomposites: A review

H. Ait Said, H. Mabroum, M. Lahcini, H. Oudadesse, A. Barroug, H. Ben Youcef, H. Noukrati

## ► To cite this version:

H. Ait Said, H. Mabroum, M. Lahcini, H. Oudadesse, A. Barroug, et al.. Manufacturing methods, properties, and potential applications in bone tissue regeneration of hydroxyapatite-chitosan biocomposites: A review. *International Journal of Biological Macromolecules*, 2023, 243, pp.125150. 10.1016/j.ijbiomac.2023.125150 . hal-04164344

**HAL Id: hal-04164344**

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Submitted on 21 Jul 2023

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# **Manufacturing methods, Properties, and Potential Applications in Bone Tissue**

## **Regeneration of Hydroxyapatite-Chitosan Biocomposites: A Review**

H. Ait Said<sup>a,b</sup>, H. Mabroum<sup>c</sup>, M. Lahcini<sup>c</sup>, H. Oudadesse<sup>d</sup>, A. Barroug<sup>b,e</sup>, H. Ben youcef<sup>a\*</sup>,  
H. Noukrati<sup>e\*</sup>

*<sup>a</sup>Mohammed VI Polytechnic University (UM6P), High Throughput Multidisciplinary  
Research laboratory (HTMR-Lab), 43150 Benguerir, Morocco*

*<sup>b</sup>Cadi Ayyad University, Faculty of Sciences Semlalia (SCIMATOP), Bd Prince My Abdellah,  
BP 2390, 40000 Marrakech, Morocco*

*<sup>c</sup>Cadi Ayyad University, Faculty of Sciences and technologies, IMED Lab, 40000 Marrakech,  
Morocco*

*<sup>d</sup> University of Rennes1, ISCR-UMR, 6226, Rennes, France*

*<sup>e</sup> Mohammed VI Polytechnic University (UM6P), faculty of medical sciences (FMS), High  
Institute of Biological and Paramedical Sciences, ISSB-P, Morocco*

### **\* Co-corresponding authors:**

#### **- Hassan Noukrati, Research, and Education Fellow**

Mohammed VI Polytechnic University (UM6P), FMS-ISSB-P

Hay Moulay Rachid Ben Guerir, 43150 Benguerir, Morocco

E-mail: [hassan.noukrati@um6p.ma](mailto:hassan.noukrati@um6p.ma)

Tel: +212 6 66 91 11 92

#### **- Hicham Ben Youcef, Professor**

Mohammed VI Polytechnic University (UM6P), HTMR-Lab

Hay Moulay Rachid Ben Guerir, 43150 Benguerir, Morocco

E-mail: [hicham.benyoucef@um6p.ma](mailto:hicham.benyoucef@um6p.ma)

Tel: +212 6 66 06 37 83

Fax: +212 5 25 07 28 70

## **Abstract**

Hydroxyapatite (HA) and chitosan (CS) biopolymer are the major materials investigated for biomedical purposes. Both of these components play an important role in the orthopedic field as bone substitutes or drug release systems. Used separately, the hydroxyapatite is quite fragile, while CS mechanical strength is very weak. Therefore, a combination of HA and CS polymer is used, which provides excellent mechanical performance with high biocompatibility and biomimetic capacity. Moreover, the porous structure and reactivity of the hydroxyapatite-chitosan (HA-CS) composite allow their application not only as a bone repair but also as a drug delivery system providing controlled drug release directly to the bone site. These features make biomimetic HA-CS composite a subject of interest for many researchers. Through this review, we provide the important recent achievements in the development of HA-CS composites, focusing on manufacturing techniques, conventional and novel three-dimensional bioprinting technology, and physicochemical and biological properties. The drug delivery properties and the most relevant biomedical applications of the HA-CS composite scaffolds are also presented. Finally, alternative approaches are proposed to develop HA composites with the aim to improve their physicochemical, mechanical, and biological properties.

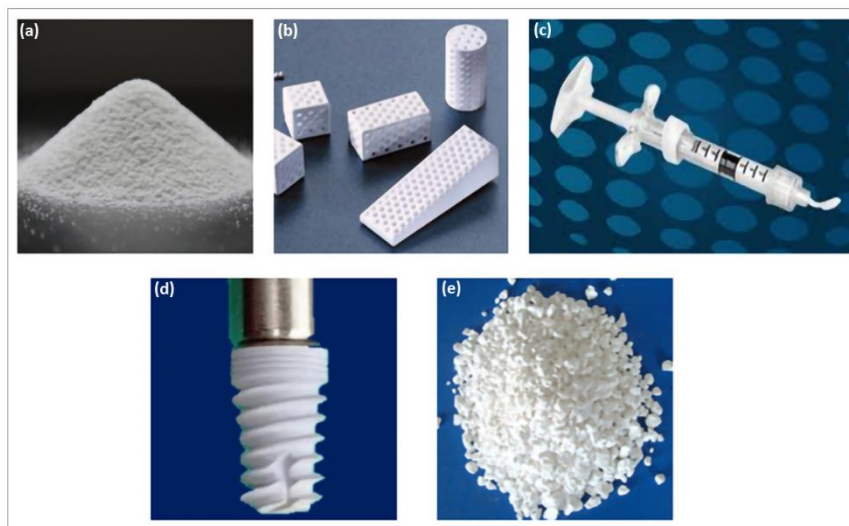
**Keywords:** Biocomposite, biomedical, chitosan, drug delivery, hydroxyapatite.

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## 1. Introduction

Nowadays, the quality of life is significantly affected by several bone disorders like bone infections, diseases, tumors, and bone fractures. Recently, progress has been achieved to treat the loss or failure of bone using healing active materials and biomaterials-based tissue engineering strategies [1]. According to statistical data, the global biomaterial market was expected to reach \$149.17 billion by 2021 rising from \$70.90 billion in 2016 [2]. Particularly, orthopedic implants based-biomaterials are highly demanded, due to the aging of the population, increasing life span, injuries, surgical procedures, infections, and diseases [3,4].

Different treatments are available to rectify bone defects [1]. The autografts bone grafts, grafting tissue from the same donor, is the gold standard for bone defects repair. They are used in about 60 % of bone graft procedures due to their capacity to promote new bone formation and growth [2,5]. Other options are used including allografts, grafting from genetically different individuals of the same species, and xenografts (grafting from other species). However, some issues such as low availability, pain, negative immune response, poor healing potential, and the risks of diseases transmission, related to these natural biomaterials can arise. All these limitations directed the research towards the development of better techniques and substitute materials. Various synthetic biomaterials, like metals, biopolymers, bioactive glasses, injectable cements, calcium phosphates, and composites have been widely used for bone substitutes [6–10]. Calcium phosphates (CaPs) materials are the vast majority of biomaterials used in biomedical applications due to their similarity with the mineral phase of natural bone. CaPs are the major inorganic constituent of bone (~ 60 wt% of bone), and the main constituent of tooth enamel (~ 90 wt%) [8]. These materials represent alternatives to natural bone grafts and exist in different forms such as powder, granule, ceramic, cement, and coating (Fig. 1).



**Fig. 1.** Different forms of calcium phosphates biomaterials: (a) Powder for the application in protective coatings, (b) blocks-based 3D Scaffold bone substitute materials for filling or reconstructing bone fractures or for filling bone defects, (c) CaP cement used as injectable materials for minimally invasive surgery, (d) coated dental implant and (e) granules used for bone repair or replacement.

They are widely used in orthopedics, dentistry, and bone regeneration due to their good biocompatibility, osseointegration, and osteoconduction. The existing CaPs compounds and their major properties are displayed in table 1. The pH stability in aqueous solutions and the solubility of these materials depend on the Ca/P atomic ratio. A lower Ca/P atomic ratio leads to a more acidic and water-soluble CaP material [8,11]. According to the solubility property, CaPs can be classified in increasing order of degradation rate as follows: Monobasic calcium phosphate monohydrate (MCPM) > Tetracalcium phosphate (TTCP)  $\approx$   $\alpha$ -tricalcium phosphate ( $\alpha$ -TCP) > Dibasic calcium phosphate dehydrate (DCPD) > Octacalcium phosphate (OCP) >  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) > Calcium deficient hydroxyapatite (CDHA) > Hydroxyapatite (HA) > Fluorapatite (FA).

Among these CaPs, hydroxyapatite (HA) is the most common phosphate material used in biomedical applications, due to its excellent stability in the physiological environment and

biological responses. It is able of integrating into bone structures being resorbed and support bone tissue in-growth without dissolving [12]. HA with its chemical composition similar to that of bone mineral, osteoconductive capabilities, and biocompatibility, is a potential material for bone repair and replacement, treatment of maxillofacial deformities, and drug delivery applications [13,14]. It belongs to the most bioactive and biocompatible materials commercially available [12,15]. However, its application in the biomedical field is currently limited to powders and coatings on metallic substrates, because of the poor mechanical properties of HA. Recent trends in biomaterials research are focused on overcoming the limitations of HA, precisely its mechanical properties, and improving its biological adaptability. The way to achieve suitable physicochemical, biological, and mechanical features is to create biomimetic compounds, inspired from the structure and chemical composition of the natural bone tissue. In fact, hydroxyapatite-polymer biocomposites proved such an approach and have shown excellent mechanical and biological properties [16]. Particular attention has been paid to hydroxyapatite-chitosan (HA-CS) biocomposites, which are promising to mimic both the inorganic and the organic part of the natural bone [8,17–20]. As a natural amino polysaccharide, chitosan (CS) has reactive hydroxyl and amino functional groups, which determine its chemical reactivity, solubility, and bioactivity [2,21]. This polymer has been largely applied in bone tissue engineering, owing to its both excellent biological properties and reactivity. Moreover, it can be used to mimic the extracellular matrix, due to its molecular structure similar to collagen. The combination of HA and CS was extensively investigated for various purposes [22,23]. The HA-CS composites biomaterials can serve not only as a scaffold for bone regeneration but also as drug delivery carriers [20,24,25].

Hydroxyapatite and its composites are the subject of intense research and several research papers have been recently published in reputable journals and conferences, due to their large application in the biomedical field, especially in bone tissue regeneration. Many reviews have

been published in the literature on HA and derivatives, including its preparation method, physicochemical and biological characteristics, and their biomedical applications [26–29]. Moreover, many authors have reviewed the use of HA in the orthopedic field, and as a versatile system for the delivery of different biologically active molecules [28,30–33]. As we are aware, no combinatory review work discussing the manufacturing technics, physicochemical features and application in bone tissue engineering of HA-CS, doped HA-CS and drug loaded-HA-CS composites was published.

The present review provides major advances in HA-CS composites and focuses on manufacturing methods, including conventional and novel three-dimensional bioprinting technology, and biological and mechanical enhancement by the polymeric phase. It highlights the role of HA-CS, doped-HA-CS and drug loaded-HA-CS composite scaffolds in bone tissue regeneration and the delivery of drugs (e.g., antibiotics, anti-inflammatory and anti-cancer), allowing the creation of a database of desired materials based on their properties, performance and targeted application. Several approaches in order to improve the physicochemical properties of the HA-CS composite are presented. This review provides easy access to relevant information on HA-CS biocomposites while.

**Table 1.** Main calcium phosphates (CaP) used for biomedical applications [8,11].



Name	Formula	Molar Ratio Ca/P	pH stability range	Solubility at 25 °C (g/L) in water
Monobasic calcium phosphate monohydrate (MCPM)	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	0.5	0.0 - 2.0	~ 18
Monobasic calcium phosphate anhydrate (MCP or MCPA)	$\text{Ca}(\text{H}_2\text{PO}_4)$	0.5	*	~ 17
Dicalcium phosphate anhydrous (DCPA) or monetite	$\text{CaHPO}_4$	1.0	2.0 - 5.5 (> 100 °C) *	~ 0.048
Dibasic calcium phosphate dehydrate (DCPD) or brushite	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	1.0	2.0 - 6.0	~ 0.088
Octacalcium phosphate (OCP)	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	1.33	5.5 - 7.0	~ 0.0081
$\alpha$ -talcium phosphate ( $\alpha$ -TCP)	$\alpha\text{-Ca}_3(\text{PO}_4)_2$	1.5	**	~ 0.0025
$\beta$ -talcium phosphate ( $\beta$ -TCP)	$\beta\text{-Ca}_3(\text{PO}_4)_2$	1.5	**	~ 0.0005
Amorphous calcium phosphate (ACP)	$\text{Ca}_x\text{H}_y(\text{PO}_4)_z \cdot n\text{H}_2\text{O}$ , n = 3 - 4.5, 15 - 20 % $\text{H}_2\text{O}$	1.2 - 2.2	~ 5 - 12	***
Calcium deficient hydroxyapatite (CDHA CDHAp; Ca-def HA)	$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$ (0 < x < 2)	1.50 - 1.67	6.5 - 9.52	~ 0.0094
Hydroxyapatite (Hap or HA)	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	1.67	9.5 - 12.0	~ 0.0003
Fluorapatite (FAP or FA)	$\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$	1.67	**	~ 0.0002
Tetracalcium phosphate (TTCP, or TCP)	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	2.0	**	~ 0.0007

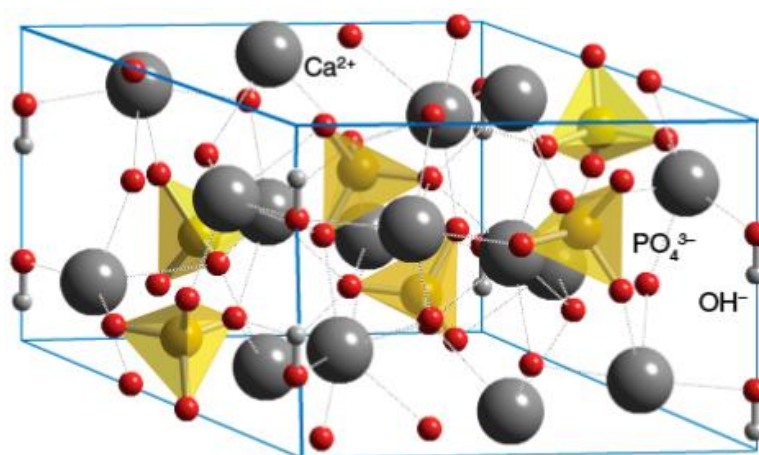
\* These compounds are stable at temperatures above 100 °C.

\*\* Cannot be precipitated from aqueous solutions.

\*\*\* The solubility cannot be measured.

## 2. Hydroxyapatite structure

Hydroxyapatite (HA) commonly known as hydroxylapatite, is a naturally occurring form of calcium phosphates that constitute the largest number of inorganic components in human bones [12]. The inorganic mineral part of bones consists of ion-substituted HA crystallites, formed by the substitution of a fraction of carbonate ions by phosphates or hydroxyl ions within the carbonated apatite structure. The chemical formula of HA is  $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$  with a Ca/P ratio of 1.67, however it is usually written as  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  to note that the crystal unit cell contains two entities of HA [15]. The unit of HA can crystallize in the hexagonal system (Fig. 2), with a space group  $\text{P6}_3/\text{m}$ , and constants  $a$  and  $c$  are equal to 0.942 and 0.688 nm, respectively. The  $\text{OH}^-$  ion can be replaced by fluoride, chloride, or carbonate, resulting in fluorapatite, chlorapatite, or carbonated apatite, respectively. About 50 % (by volume) and 65 wt% of the human bone mineral is a modified form of HA [34]. The main mineral of dental enamel and dentin is composed of carbonated calcium-deficient HA [35]. The crystals of HA can also be found in the small calcifications, the pineal gland, and other structures named corpora arenacea, or brain sand.



**Fig. 2.** Structure of hexagonal hydroxyapatite (space group  $\text{P6}_3/\text{m}$ ) [8].

### 3. Preparation methods of Hydroxyapatite

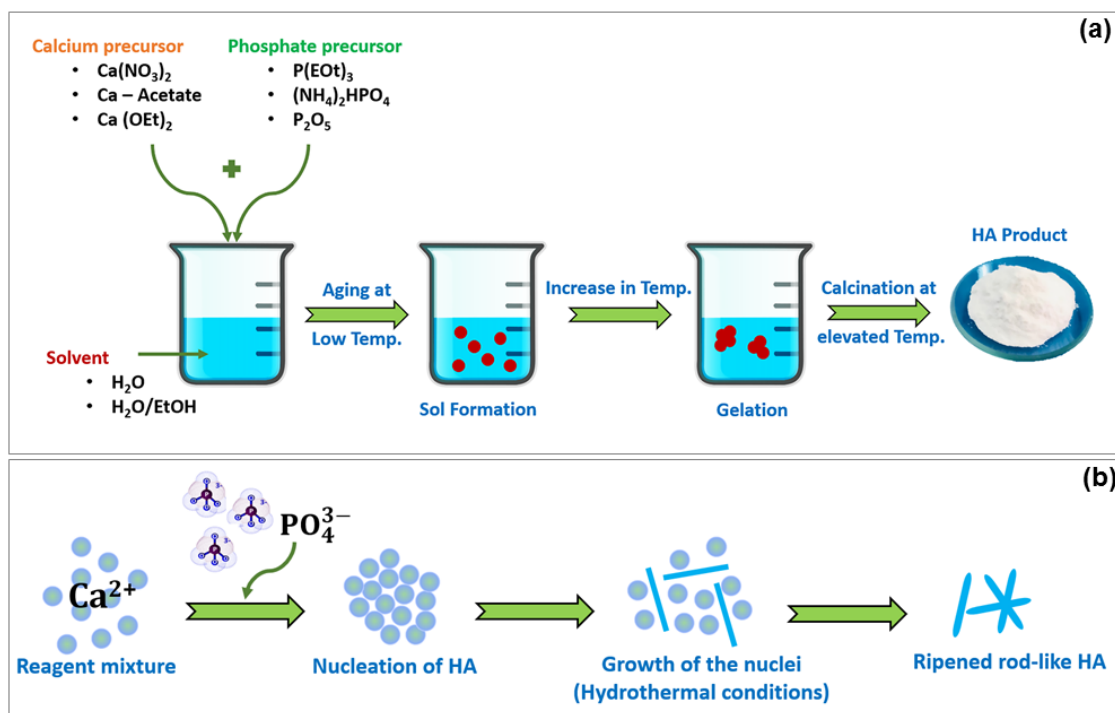
As a biomaterial, the *in vitro* and *in vivo* responses of HA are significantly affected by its preparation/synthesis techniques, consequently its physicochemical properties [36–38]. HA particles of different morphologies have been prepared in a large scale with reproducible size, tailored composition, and structure. Recently, environmentally friendly methodologies, including hydrothermal processing, template synthesis, biomimetic synthesis, and molten-salt synthesis have been investigated as viable synthesis techniques for the preparation of a range of CaP materials [15,31,39,40]. HA can be prepared by a variety of dry techniques (solid-state reactions and mechanochemical); wet-chemical techniques (hydrolysis, precipitation, hydrothermal, and sol-gel); and high-temperature synthesis (spray pyrolysis, combustion, and thermal decomposition). The HA crystals can be also obtained from naturally available bio-wastes such as mammalian bone, marine sources, plants and algae, and shell sources, due to their cost-effectiveness [40–42].

#### 3.1. Dry process

HA can be obtained using dry technics either by solid-state synthesis or mechanochemical process. In the solid-state preparation, the started precursors (phosphate and calcium) are mixed and calcined at high temperatures ( $\approx 1000$  °C) [43]. During the mechanochemical method, the started materials are ground, and then the mechanical energy is used to promote the structural changes and the chemical reactions. This process is often used for the synthesis of calcium phosphate-based ceramic materials (HA, TCP, ...) and biphasic calcium phosphate (BCP). In contrast, it cannot be used for the preparation of composites based on CaPs and organic molecules, due to the high temperatures used during manufacturing. In addition, high-temperatures sintering used in this technique prevents the association with drug molecules during synthesis.

### 3.2. Wet methods

The wet processes are based on the chemical reactions between calcium and phosphate precursors in solution, which are carried out using different solvents and at different temperatures. In this preparation route, sol-gel, hydrothermal, hydrolysis, and *in situ* precipitation (chemical precipitation) are the main techniques studied [15,40]. Using the sol-gel method (Fig. 3a), the precursor monomers are mixed and converted into a solution, which is a colloidal suspension of solid particles [44,45]. In this process, the solution is then aged, gelled, dried, and calcined to remove the residues of the organic phase. The hydrothermal method can be considered as high-temperature chemical precipitation (Fig. 3b). During this process, organic additives such as acid or basic solutions are often used to control the structure and morphology of the resulting crystals. Using the hydrolysis process, HA particles are obtained through the transformation of another calcium phosphate phase in an aqueous solution. Concerning the emulsion synthesis, it can be performed in an oil-in-water emulsion, water-in-oil emulsion, or water-in-oil-in-water double emulsion using different surfactants [40]. The use of this technic inhibits the formation of the agglomerates and allows controlling the morphology and size of the obtained particles. Chemical precipitation is a simple technic of HA preparation using calcium and phosphorus precursors [20,46]. The reactants are mixed under controlled conditions (i.e., temperature and pH) and subjected to aging and precipitation. Finally, the resulting precipitate is washed, filtered, and dried. Precipitation of HA crystals can occur using various calcium (e.g.,  $\text{Ca}(\text{NO}_3)_2$ ,  $\text{CaCl}_2$ ,  $\text{Ca}(\text{OH})_2$ , and  $\text{CaCO}_3$ ) and phosphorus (e.g.,  $(\text{NH}_4)_2\text{HPO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$ , and  $\text{H}_3\text{PO}_4$ ) precursors [19,20,47–50]. The phosphate and calcium sources, the Ca/P ratio, temperature, pH, precipitation time, aging time, and drying method can be varied to control the characteristics of the final product [51–53].



**Fig. 3.** Schematic representation of hydroxyapatite preparation using : a) Sol-Gel and (b) hydrothermal methods.

### 3.3. High-temperature processes

The high-temperature synthesis is usually based on the burning of part or the totality of the starting precursors [15,40]. This method includes two main processes, combustion, and pyrolysis. The combustion reaction is a one-step process creating a product of high purity [54–56]. In this technique fuels like citric acid, urea, glycine, and hydrazine are used; which require an energy supply to complete the combustion reaction, after ignition at a low temperature. During the pyrolysis process, the product particles are formed in a gas phase generated by the evaporation of the starting liquid precursors [57–59]. In fact, the reactants are sprayed into a flame of an electric furnace using an ultrasonic spray generator.

### 3.4. Extraction of hydroxyapatite from biological sources

Natural sources or wastes such as mammalian bone, marine or aquatic sources, shells, mineral sources, and plants are being largely explored for the extraction of biological HA [31,41,60,61].

Table 2 represents the main sources and examples of methods used for the extraction of natural HA. The use of fish scales and bones for the production of HA has significantly raised due to the increased consumption of fish products around the world, which allows the diminution of solid wastes in the fisheries industry [61–63]. These natural wastes are rich in carbonate, phosphate, and calcium, making them a great source for HA production. Mammalians are also considered important sources for the preparation of HA particles. Among these sources, the extraction of HA from bovine bones was widely reported compared to other sources like horses, porcine, and camels [42,64]. The cortical fraction of the femoral bone is the most used due to its morphological and structural similarity to human bone. Many properties such as particle size, Ca/P ratio, shape, and crystallinity of the extracted HA, depend strongly on the applied extraction technic and thus processing parameters (i.e., pH, calcination temperatures, and solvents). Most of the extraction methods from mammalian sources used the calcination process or a combination of calcination with other technics. The shells of eggs, clams, and cockles are abundant in nature and are widely investigated in the literature [31,65,66]. They are very rich in calcium carbonate ( $\text{CaCO}_3$ ) which can be converted to HA. Eggshell, for example, contains about 94 %  $\text{CaCO}_3$  and can be used as starting precursor of Ca for HA synthesis. Among the natural mineral sources, limestone is one of the minerals used for synthesizing HA, due to the Ca amount in its structure. Limestone is naturally formed from the chemical precipitation of animal skeletons or shells, and algae or foraminifera, which are rich in  $\text{CaCO}_3$  [42,67,68]. Its abundance makes it one of the most exploited calcium sources for the preparation of HA.

Generally, all the natural sources are usually treated before starting the extraction process [60,67,69]. The pretreatment includes washing and hitting to remove the fats, dirt, organic proteins, and other components like soft tissues and bone marrow. The use of these biological sources results in HA particles with different characteristics [63]. The extracted HA particles are frequently obtained in the form of a needle or rod-like morphology [42,70]. The crystallinity

of the HA depends significantly on the extraction method. The use of the precipitation technique results in low crystallinity of HA particles, whereas the thermal process increased the crystallinity degree. On the other hand, the reported results demonstrate that the Ca/P ratio is non-stoichiometric for the majority of the natural sources [63]. In the case of aquatic/marine and mammalian sources, the presence of some trace elements might increase the Ca/P ratio to values higher than those observed for stoichiometric HA. For other sources such as algae, minerals, and shells, the presence of calcium species like CaO may increase the Ca/P value.

Even though several techniques have been attempted for the synthesis of HA, only a few of them are fulfilling both the cost-effectiveness and performance requirements. The main obstacles of most of the used techniques include not only the diverse solvent, surfactant, or precursor materials needed during the preparation, but also the expensive and complicated processes, phase impurities, and agglomeration issue. Worldwide, a variety of preparation routes have been applied for the fabrication of HA. Attempts have been made to control several properties like crystallinity, geometry, stoichiometry, size, and particle agglomeration by employing new synthesis methods or via modification of preexisting methods. The combination of two or more of these methods was tried like combinations of hydrothermal-hydrolysis, hydrothermal-microemulsion, or hydrothermal-mechanochemical, in order to obtain HA with improved properties. Other strategies being investigated include physical blending, coating, and freeze-drying. For instance, *in situ* precipitation is the most used method for CaP production due to its versatility, cost-effectiveness, reproducibility, and homogeneity of HA crystals obtained.

**Table 2.** Natural sources and extraction methods of hydroxyapatite.

Source	Extraction method	Ref
Mammalian: bovine, camel, horse, pig	Calcination, alkaline hydrolysis, hydrothermal, combination of methods	[42,64,69,71,72]
Marine/aquatic: fishbone, fish scale	Calcination, alkaline hydrolysis, hydrothermal, combination of methods	[42,61–63]
Shells: cockle shell, seashell, eggshell, clamshell, mussel shell	Sol-gel, precipitation, mechanochemical, Microwave, Calcination, hydrothermal, combination of methods	[41,42,60,66,70,73]
Plants: stalk, leaves, and flowers, Fruits, peel, woods, algae	Microwave irradiation, pyrolysis, hydrothermal, chemical precipitation, calcination, alkaline hydrolysis, a combination of methods	[42,60,74]
Mineral: limestone	Calcination-precipitation, calcination-ultrasonic irradiation, Calcination-hydrothermal	[42,67,68]

#### 4. Requirement from calcium phosphates for biomedical applications

Calcium phosphates are commonly used in the biomedical field in the form of granules, coating, cement, paste, and scaffold. To be applied successfully, a variety of characteristics (physical-chemical, mechanical and biological properties) may be required [8,75]. Some properties are unavoidable; biocompatibility, a guarantee of acceptance by the body is one of them. The majority of CaPs materials are biocompatible due to their abundance in the body in either dissolved or solid form. A critical drawback that hampers wider medical applications of CaPs, especially HA, is their low mechanical properties [15]. Therefore, current attempts are focused on the development of biocomposites based on HA and biopolymers in order to overcome these limitations and even improve their biological adaptability.

The incorporation of polysaccharides like the biopolymer chitosan (CS) into the HA matrix has been proposed as an appealing approach to enhance HA performance. Researches proved that



such biocomposites had excellent mechanical and biological properties compared to their separate use [17,19]. Hydroxyapatite-chitosan (HA-CS) composites can serve as both a scaffold for bone regeneration and a drug delivery carrier [20,21].

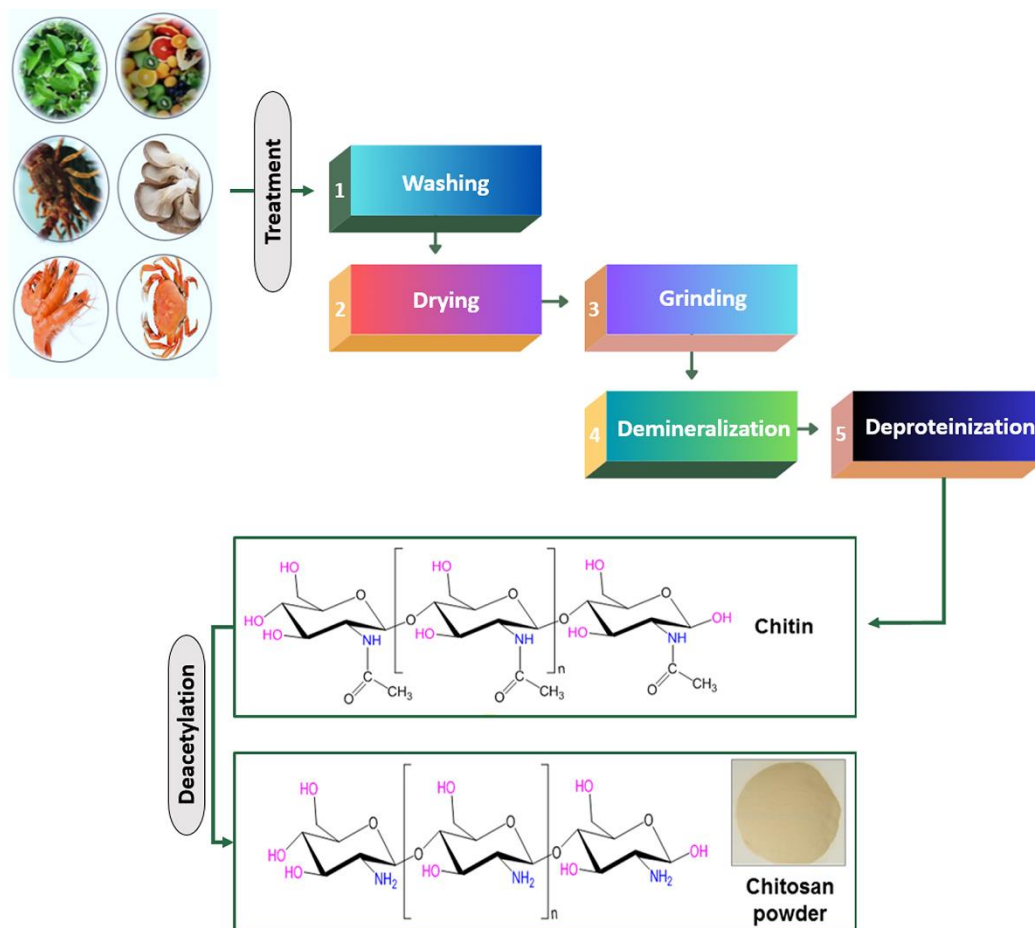
## 5. Chitosan

Chitosan is a polysaccharide derived from chitin (Fig. 4), the second most abundant polysaccharide in the world, after cellulose [76]. Chitin is found in many natural organisms such as the exoskeleton of sea crustaceans (shrimp and crab), yeast, and some bacterial and fungal cells. Its chemical structure is made up of (1→4)-2-acetamide-2-desoxy-β-D-glucopyranose (Fig. 4). Chitosan is a linear homopolymer constituted of β-(1,4)-linked N-acetyl-glucosamine units. It is a partially deacetylated polysaccharide acquired from the basic deacetylation of chitin, through a chemical or enzymatic reaction. When the deacetylation degree of chitin reaches about 50 %, it is called chitosan and becomes soluble in acidic media [77,78]. The extraction of chitosan from different sources is schematically presented in Fig. 4.

The performance of chitosan depends on the source and the deacetylation degree of chitin and its extraction method [79]. Properties like deacetylation degree, functional groups, crystallinity, and solubility, of chitosan are significantly affected by the processing conditions. The amino and hydroxyl groups of chitosan allow it to be a highly reactive polysaccharide. It can contribute to electrostatic interactions with negatively charged organic molecules, due to its cationic character in acidic solutions. Chitosan is also distinguished by its variable molecular weight which makes it a promising biopolymer for various application fields, as it has an impact on its physicochemical and biological characteristics [77,80]. The molecular structure of chitosan provides good thermal and chemical stability. Depending on the source and preparation procedure, CS molecular weight can range from 300 to 1000 kDa [18].

Chitosan has been widely used in tissue engineering, due to both excellent reactivity and biological properties like biocompatibility, biodegradability, nontoxicity, bioactivity, and antimicrobial property. These properties make chitosan a promising biopolymer for various applications in the biomedical field. The use of chitosan has been proven to improve cell adhesion and proliferation [81,82]. The incorporation of this polymer into the matrix of many inorganic materials can enhance the proliferation of osteoblast cells and promote bone fracture healing [81,83]. Furthermore, it was reported that the addition of this polysaccharide to the CaP formulations and other compounds can improve their mechanical properties and drug delivery ability, which are crucial for tissue regeneration and bone repair [84,85]. Chitosan components with intrinsic antimicrobial properties can be made into porous scaffolds. The antimicrobial activity of the scaffolds can be further improved by adding other therapeutic agents. In addition, chitosan has remarkable healing properties, as it increases the rate of healing of open wounds by stimulating cell growth and tissue repair in surgical wounds [79].

Nevertheless, chitosan is not as strong as inorganic materials and shows weak mechanical properties, limiting its application as a bone substitute. Another limitation of this polymer is its low solubility in neutral or alkaline solutions. Several studies have been performed on CS modification through different techniques like functionalization, grafting, or polymerization to achieve newer CS derivatives with superior features over the homopolymeric CS [86]. On the other hand, combining the physicochemical and biological properties of the chitosan phase with the inorganic characteristics attracted a growing interest to create new inorganic-organic biocomposites that exhibit simultaneously biomimetic capacity and improved mechanical properties when compared to the separate components. In fact, researchers have expressed a great interest in developing biocomposites based on hydroxyapatite and chitosan (HA-CS) biomaterials for bone tissue engineering and as a drug delivery system [21].



**Fig. 4.** Extraction of chitosan from different natural sources.

## 6. Hydroxyapatite-Chitosan composite

Skeletal bones consist mainly of carbonate substituted HA and organic phase (predominantly type collagen I). Thus, an orthopedic implant produced from similar components is likely to mimic the natural bone. Over the past decades, CaPs ceramics, particularly HA were the most common biomaterial used as bone graft substitutes, due to their chemical similarity with the mineral phase of bone and unique biological properties. However, their clinical applications in non and low-load-bearing sites have been limited because of their brittleness. Many efforts have been made to develop CaP-containing composite biomaterials, especially HA-polymer biocomposites.

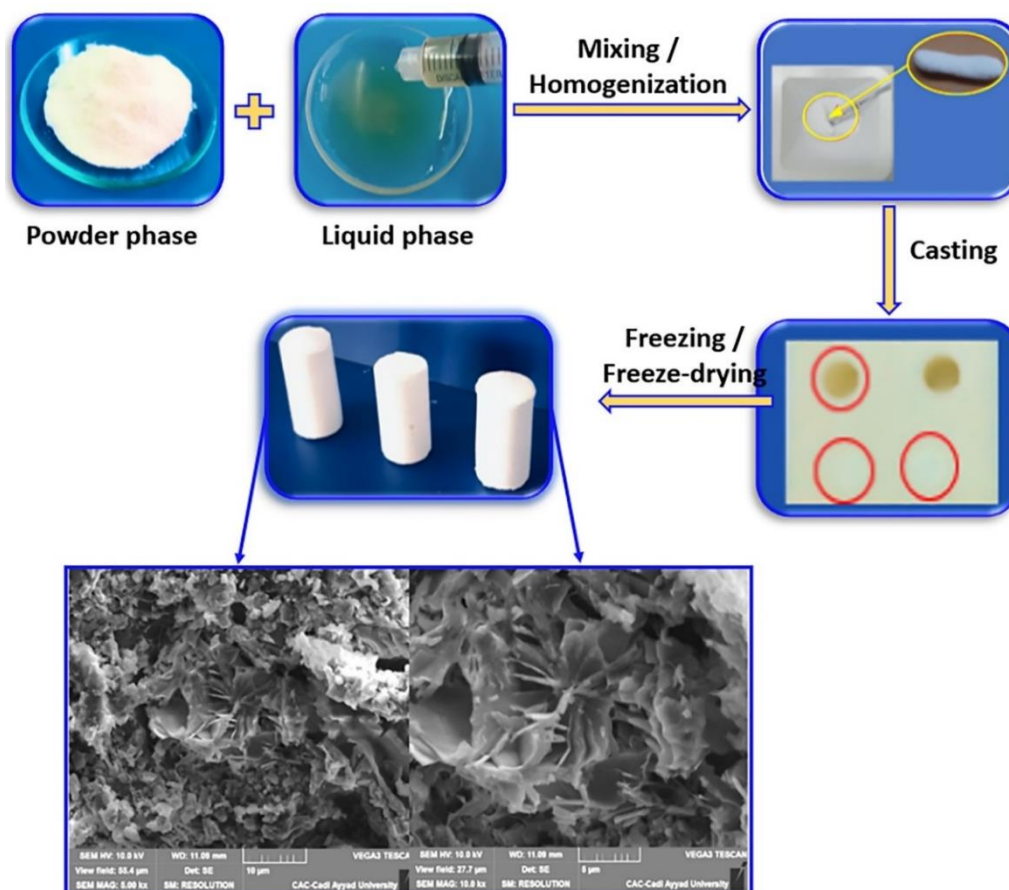
The HA surface provides effective nucleating sites to create an apatite layer in contact with the body fluids and culture medium, due to its high stability among other CaPs ceramics [12]. Furthermore, the physicochemical, biological responses, and solubility of HA can be controlled by chemical functionalization [87]. Thus, HA is largely used to prepare HA-polymer biocomposites usually with the aim to enhance its mechanical properties as well as its biological properties (i.e., biodegradability, osteoconductivity, and bioactivity) [16]. It is considered that collagen/HA biocomposite is one of the most promising bone graft biomaterials, due to its ability to mimic both the composition and structure of natural bone [88–92]. Considering the spatial relationship between inorganic and organic phases, new processes like biomineralization, and self-assembly have been recently applied to produce collagen/HA biocomposites with hierarchical structures [89,91]. Several investigations have been carried out on another biopolymer, namely chitosan (CS) polymer, as a component of HA-containing biocomposites. The combination of HA and CS increased the mechanical behavior and improved the biological properties of the HA-CS composite matrix, compared to the separate components [19,20,93].

### **6.1. Fabrication techniques**

Previous research, regarding the applications of biocomposites based on HA and chitosan, was mainly focused on the preparations methods [5,18–20]. The HA-CS composite was extensively studied for orthopedics and maxillofacial surgeries. In fact, it was used either to increase the alveolar edge, to fill bone defects, as a fusion of the spine vertebrae, middle ear implants, or as a coating for the metallic prosthesis. The choice of the manufacturing procedure of HA-CS composite depends on different criteria, including the area application, the nature of the reagents used as precursors, the nature of polymeric phase/content, pH, temperature, Ca/P ratio, liquid to solid ratio, and size requirement.

### 6.1.1. Mixing method

The simple mixing technic of HA and CS is one of the most common approaches used for the preparation of HA-CS conventional scaffolds for bone tissue engineering. The method is based on mixing of previously prepared HA powder with the CS solution under stirring to generate homogeneous suspensions or pastes (Fig. 5) [20,94]. Porous scaffolds (Fig. 5) are usually generated from the obtained pastes using a freeze-drying process. The resulted HA-CS biocomposites by freeze-drying process provide a suitable environment for cell adhesion and promote bone regeneration, due to the appropriate pore size distribution and high specific surface area. Ying et al. [95] synthesized hydroxyapatite-chitosan scaffolds with different HA/CS ratios (10/90, 20/80, and 70/30) via a freeze-drying technique. A colloidal solution and deionized water were added into a CS/acetic acid solution under stirring. The homogenized mixture was ultrasonicated to remove the eventual air bubbles; then, the system was freeze-dried. In order to remove the excess of acetic acid, the composites were soaked in a NaOH solution, washed with deionized water, and freeze-dried again. The resulting composite showed a better distribution of the apatite particles within the polymer matrix and excellent porosity ( $79.48 \pm 1.09 - 83.39 \pm 1.01$  %).



**Fig. 5.** Example of hydroxyapatite-chitosan composite fabrication through freeze-drying technique, and the surface morphology of the final products using SEM analysis.

In order to promote nucleation/growth and reduce the reaction time, microwave radiation was exploited [40,96]. This approach was used to generate nanostructured compound systems of HA and CS. The HA apatite was first precipitated from  $\text{CaCl}_2$  and  $(\text{NH}_4)_2\text{HPO}_4$  precursors, in an alkaline medium ( $\text{pH} \approx 10.0$ ), then the obtained HA powder was dispersed in solutions at different CS contents (5 and 10 wt%) in acetic acid followed by microwave irradiation.

### 6.1.2. Evaporation casting method

Casting and evaporation or dry-casting methods involve the evaporation of a solvent or a mixture of solvents from an initial polymeric solution resulting in a membrane, via precipitation [97–99]. A slurry of precipitated HA was added dropwise to CS acetic acid solution (2 wt.%) and the homogeneous mixture was poured into a glass dish and spread to create a homogeneous

film, followed by a drying step. [98] used the casting/solvent evaporation method to generate genipin cross-linked chitosan/HA composite membranes. A powder of HA was added to a chitosan-genipin-acetic acid solution, then the obtained homogeneous HA/chitosan-genipin suspension was casted into a glass Petri plate and dried.

### **6.1.3. Coating method**

Implants for high-load-bearing applications, such as in the case of femur and hip replacement devices, should have sufficient mechanical features and resistance to wear and corrosion [100,101]. Several metals like titanium (Ti), Ti and cobalt alloys, and stainless steel, exhibit excellent mechanical performance, attributed to their high strength/weight ratio, especially in the case of Ti metal and alloys. However, implant failures, mainly attributed to aseptic loosening or bone infection after implantation, are frequently present [102]. Therefore, coating of the implants using apatitic CaP or composite provides a highly bioactive interface for supporting the bone tissue.

Composites based on calcium phosphate and chitosan polymer can be prepared as coatings through electrochemical procedures, allowing the deposition of HA particles and CS macromolecules. The coating method, known also as electrochemical deposition, is commonly used for preparing hybrid biomaterials for biomedical applications. The composition of the deposit can be controlled by varying the chitosan or HA content in the deposition bath. Recently, CS-HA composite was developed by Pang et al. [103] using an electrophoretic co-deposition of CS-HA composite to enhance the corrosion resistance of 316L stainless steel in contact with the physiological environment. Electrochemistry-assisted reacting deposition technic was employed by Huang et al. [104] to create porous CS-HA scaffolds. Redepenning and coworkers [105] also prepared HA and CS composites using an electrochemical process, starting from CS and brushite, to coat titanium substrates. The brushite-chitosan composite was converted to hydroxyapatite-chitosan in an alkaline medium of sodium hydroxide. Qualitative

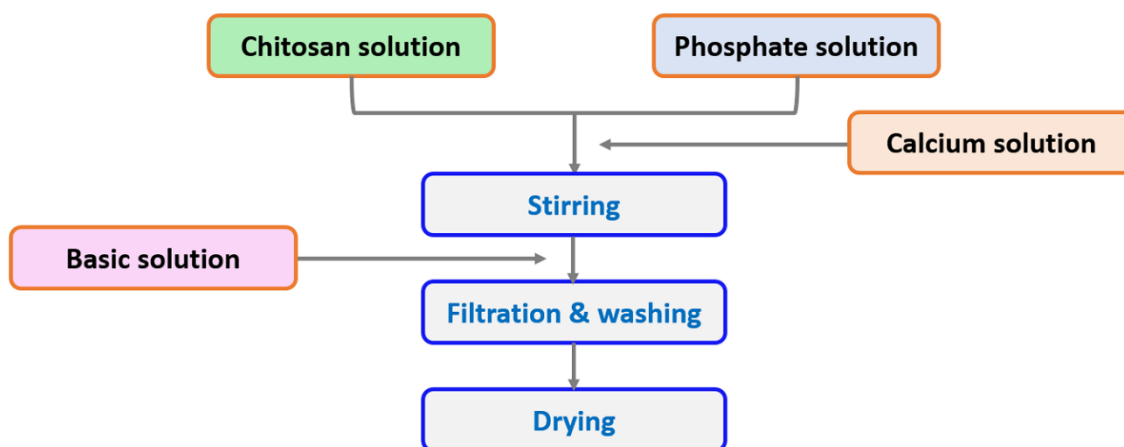
assessment of the resulted coatings showed that the cells adhesion was significantly improved compared to electrodeposited coatings of HA alone. Additionally, remarkable properties of this method were proved by using materials such as Biovetir II, coated with CS-HA composite to enhance the implant interaction toward cells, integration into the subcutaneous tissue, and biodegradability [106].

CaP-polymer biocomposites face various limitations criteria, and restrictions when used as coatings agents. Although commonly used deposition strategies have been found efficient *in vitro* and few of them are commercially available, the obtained composite scaffolds are not yet clinically used. An ideal implant coating must meet some specific requirements such as biocompatibility, bioactivity, adequate surface roughness, porosity, cell adhesion, structural integrity, chemical and mechanical stability, and enhanced osseointegration property. Meanwhile, the coating agent must be gradually degraded during the implant integration and throughout the bone healing process. However, the degradation process of coatings implants in widely investigated simulated body fluids can be completely different than in human body conditions.

#### **6.1.4. *In situ* precipitation**

*In situ* precipitation method is a powerful strategy for the synthesis of CaPs materials and composites based on CaPs and polymers [19,20,23,107]. This method is one of the most efficient techniques used for HA-CS composite production, due to its simplicity, cost-effectivity, reproducibility, and homogeneity of the resulting product. The reactants are mixed with the chitosan solution at desired temperature and pH conditions, followed by aging and precipitation. The formation of the apatite matrix can occur using various calcium ( $\text{Ca}(\text{NO}_3)_2$ ,  $\text{CaCl}_2$ ,  $\text{Ca}(\text{OH})_2$ , and  $\text{CaCO}_3$ ) and phosphorus ( $\text{NH}_4\text{H}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$ , and  $\text{H}_3\text{PO}_4$ ) precursors [19,20,47–49]. An example of the experimental procedure used for the preparation of HA-CS is given in fig. 6.





**Fig. 6.** Preparation steps of hydroxyapatite-chitosan using *in situ* chemical precipitation.

Yamaguchi et al. [108] synthesized the chitosan/hydroxyapatite composite, at alkali pH, by dropping a mixture of phosphoric acid ( $\text{H}_3\text{PO}_4$ , 8.5 wt%) and chitosan solution in acetic acid (3 wt%) into calcium hydroxide ( $\text{Ca}(\text{OH})_2$ , 0.5 M) suspension. The resulted composites were proved to be mechanically flexible. Zima et al. [19] have developed a stepwise co-precipitation approach at room temperature to create HA-CS materials in the form of granules (300 - 800  $\mu\text{m}$  in diameter). In this method, a phosphoric acid solution was introduced directly into the chitosan solution, and afterward, the mixture was added dropwise to the  $\text{Ca}(\text{OH})_2$  suspension. During the preparation, the pH value of the mixture was adjusted to about 11 using ammonium hydroxide solution. The obtained HA-CS composites (CS content of 17 and 23 wt%) possessed good homogeneity, excellent mechanical strength, and chemical stability in a simulated body fluid. Another concept was proposed by Chen et al. [109] who mixed a homogeneous chitosan solution with an alcohol solution of encapsulated icariin (chemical compound classified as a prenylated flavonol glycoside), followed by adding  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  and  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  solutions. Afterward, microspheres of CS-HA composite were generated through the high voltage static microcapsule forming system.

Other manufacturing methods might be found in the literature; for example, a hydrothermal hot-pressing (HHP) technique was used to produce HA-CS composite from previously

synthesized DCPD-chitosan biocomposite [110]. Similarly, HA-CS biocomposites were prepared by a hydrothermal process from natural CaCO<sub>3</sub>/CS composite of crab shells [111]. HHP is a processing technique for generating a bulk ceramic body at temperatures less than 300°C [112,113]. The compression of inorganic or composite materials under hydrothermal conditions allows their densification. This process has the potential to be used for producing biocomposites with good mechanical strength via compressing water-containing powders of inorganic materials to consolidate them by squeezing out the water and compacting the particles. Several authors have demonstrated that the HA-CS composites synthesized via the co-precipitation method are well known as osteoconductive biomaterials [23]. Furthermore, this manufacturing method possesses several advantages compared to many of the techniques reported in the literature. The HA-CS synthesis using precipitation method generally occurs at room temperature; this fact greatly extends the applicability of this technique to the fabrication of biomaterials combined with thermally unstable organic substances such as proteins, antibiotics, anti-cancers molecules, and growth factors, or even cells without any thermal decomposition or loss of functionality during the manufacturing process. Escobar-Sierra et al. [23] investigated the effect of the manufacturing technique on the HA-CS properties for bone tissue engineering applications. In fact, the scaffolds produced by *in situ* precipitation have a better morphology (i.e., high porous structure and interconnected pores) and good biological properties compared to those obtained using a simple mixing process. The scaffolds with CS and HA fabricated by *in situ* protocol are potential candidates for applications in bone tissue engineering. Minocycline loaded-HA-CS composite was prepared through co-precipitation technic [114]. The developed composite showed excellent compressive strength biocompatibility.

CaP-organic-based composites have shown successful outcomes by reducing drawbacks associated with the use of individual CaP. However, the conventional techniques used to

fabricate 3D scaffolds with a porous structure have limitations to better control the architectures and pore connectivity. The key in developing CaP-polymer composites for medical applications is to mimic the complex architecture of human bone tissue. Currently, three-dimensional (3D) printing has become an important tool in the field of tissue engineering, and its further development will lead to completely new clinical possibilities.

#### **6.1.5. Three-dimensional bioprinting**

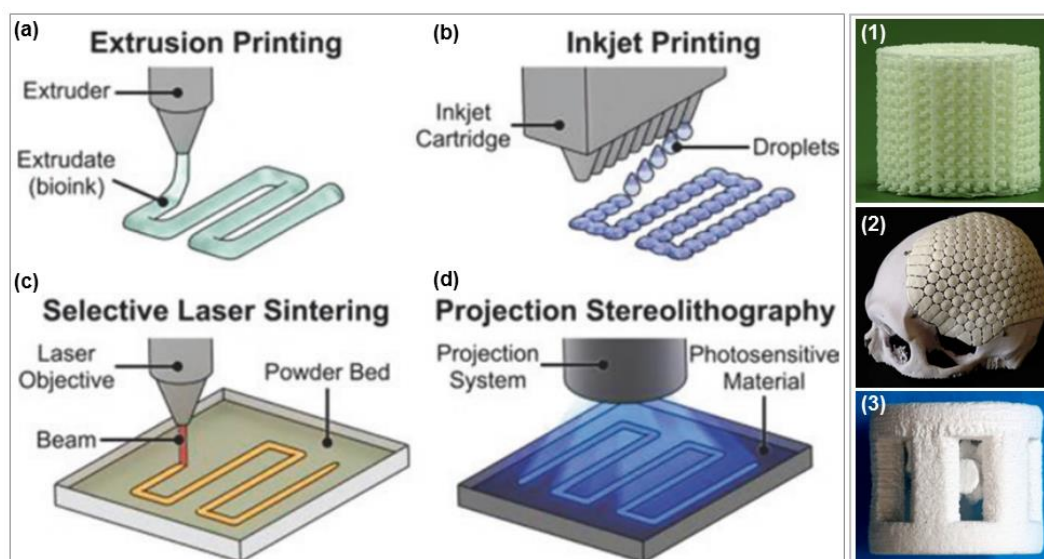
In the orthopedic field, three-dimensional (3D) bioprinting or additive manufacturing technologies are one of the most promising future trends to acquire target biomaterial properties in a desired and complex structure. 3D bioprinting is the layer-by-layer deposition of biological material with high structural complexity and design flexibility (Fig. 7). This technology has undergone significant development and is currently used in a wide range of application domains [115–117]. It can be applied to both organic and inorganic materials [114,115]. The ability to create scaffolds with controllable characteristics (internal architecture, porosity, and interconnectivity) make it highly desirable in comparison to conventional fabrication processes, which lack repeatability between the obtained scaffolds. 3D bioprinting of biologically relevant biomaterials has important advantages compared to previous strategies as it allows for the deposition of biomaterials and cells, precisely in 3D space to better mimic the native tissues. Different bioprinting systems are available; they can be inkjet-based, extrusion-based, or laser-based (Fig. 7) [115,116]. This technique consists of the deposition of biomaterials or living cell bioink droplets onto a support through a computer-designed program to create organs or human tissues.

The fused-Deposition Modeling method is the oldest 3D printing technology employed in rapid prototyping, modeling, and fabrication [118,119]. This method plays a crucial role in enhancing dimensional precision, quality of products, reduced manufacturing times, and cost of production. It is based on the deposition of molten thermoplastic in thin layers, which serves as

a support for hydrogel-based bioinks. Bioplotting is another 3D additive manufacturing technique used in biofabrication and tissue engineering [119,120], giving interesting and new possibilities for biofunctional materials fast prototyping due to its versatility. This approach is based on a high-energy light source or long-wavelength laser. In such types of printers, different syringes can be used to extrude materials in tubes or spheroids shapes. Inkjet printing, also known as drop-on-demand printing, drop-on-demand bioprinting, or drop-by-drop still holds promise for 3D printing of biomaterials and biomedical applications [9,121]. This method is a noncontact reprographic approach based on the deposition of natural or synthetic bioink droplets. Extrusion bioprinting or the direct writing method is one of the most used 3D printing methods for biomedical applications [122,123]. The bioink is continuously extruded as a strand in a controlled manner by mechanical force, typically through a screw or piston or by the use of pressurized air or pneumatic gas.

Additive manufacturing techniques could be used for the preparation of different materials including CaPs, ceramics, and composites. These biomaterials have to be easier to shape into 3D porous scaffolds with controlled pore size and highly interconnected pores, to promote cell adhesion, proliferation, migration, new tissue ingrowth, sufficient nutrient diffusion, and vascularization [123,124]. Besides, suitable biomaterials must be mechanically stable and maintain contrast integrity after printing [125]. Biocomposite scaffolds based on hydroxyapatite and chitosan were successfully produced by additive manufacturing techniques [123,125–128]. Demirtaş et al. [125] fabricated a chitosan-hydroxyapatite composite for bone tissue engineering using chitosan-hydroxyapatite hydrogel as a bioink. The obtained scaffolds exhibited excellent biological and mechanical properties. A direct ink writing process was used to produce 3D HA-CS scaffolds with a specific shape and micro-architecture for bone tissue regeneration applications [123]. In this study, the 3D printing was coupled with a freeze-drying process to mimic the natural bone in terms of porosity and interconnectivity. HA-CS based

hydrogel composite with optimized properties manufactured extrusion-based 3D printing for application in bone regeneration [126]. HA-CS composite scaffolds were manufactured by Chavanne et al. [128] through 3D printing (using Z-Corp, Z-510 system) for bone implantation and replacement. The developed composite scaffolds exhibited excellent properties in terms of porosity, pore size, mechanical properties and biocompatibility. The physicochemical and biological characteristics of the obtained scaffolds will be presented thereafter (section 7). Zafeiris et al. [127] produced HA-CS hybrid scaffolds through 3D printing process, several processing parameters such as flow, infill and perimeter speed in order to create scaffolds with desired geometry and architecture, while applying very low printing temperature (15 °C). In this study a natural crosslinking agent, genipin, was used to improve the mechanical properties of the scaffold for application in the regeneration of damaged and defected tissue.



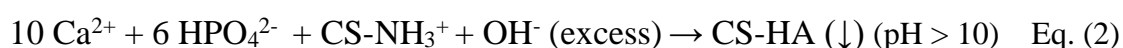
**Fig. 7.** Tri-dimensional bioprinting techniques commonly used for biomaterials manufacturing: a) Extrusion printing, b) inkjet printing, c) selective laser sintering, and d) stereolithography [119]. Examples of designed and printed scaffolds: (1) 3D printed scaffolds made of PCL/TCP composite. Reproduced with permission from [129]; (2) dicalcium phosphate anhydrous (DCPA) implant for cranial bone defects repair, and (3) 3D printed scaffolds from DCPA [8].

## 6.2. Mechanism of hydroxyapatite-chitosan composite formation

The precipitation mechanism of the HA crystals in the presence and absence of the CS polymer has been largely investigated [19,97,130,131]. During the synthesis of the HA-CS biocomposites, different chemical interactions can occur between the hydroxyl and amino functional groups of the polymer and the active sites of the HA particles. The CS polymer can exist in a solid or soluble form depending on the pH medium, due to the presence of amino functional groups in its structure. In acidic solutions, CS-NH<sub>3</sub><sup>+</sup> could be formed through protonation of the free amino groups (CS-NH<sub>2</sub>) according to the following equation:

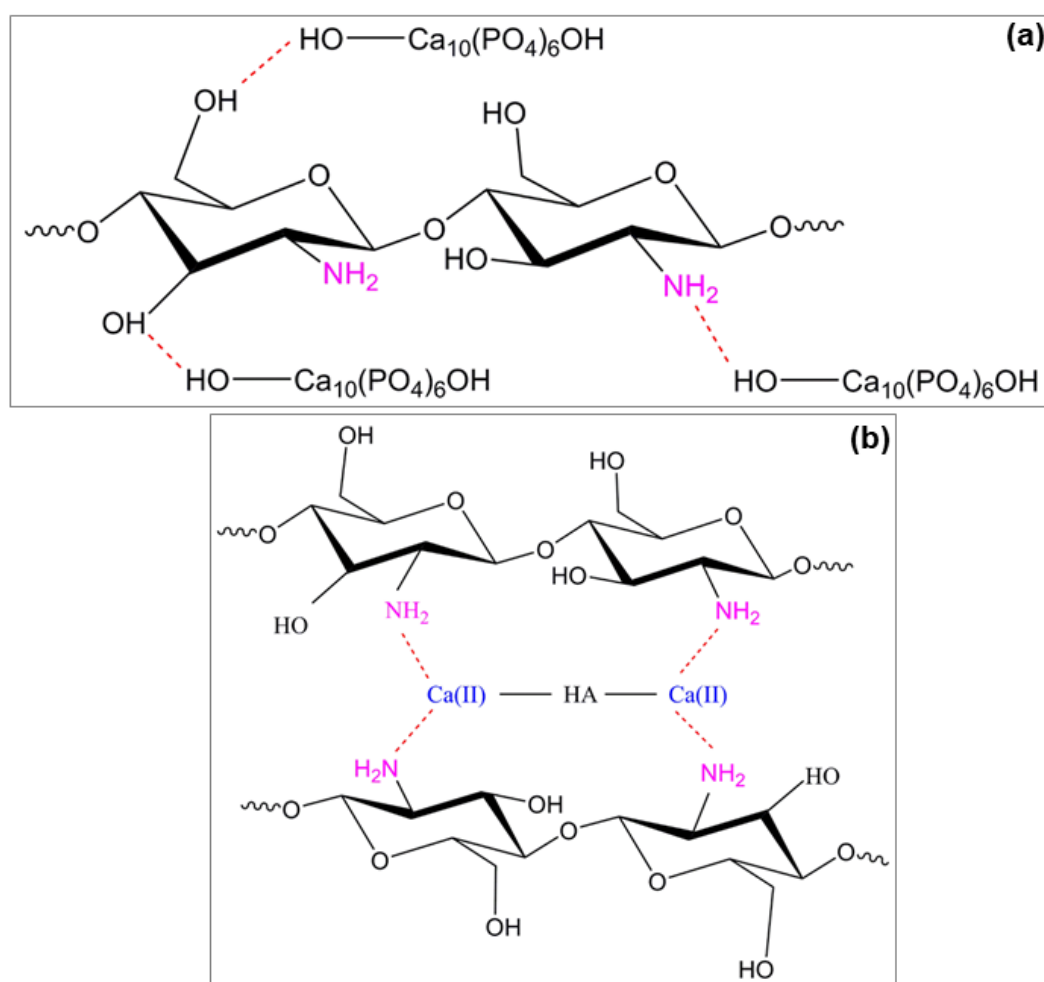


Moreover, it was reported that HA apatite is stable and can precipitate at alkaline conditions (pH > 10) or even at acidic pH (4 - 6) using the precipitation-hydrolysis process [47,51,132]. HA crystals can precipitate simultaneously in the presence of CS solution at a basic pH as follows:



Xianmiao et al. [97] performed a Fourier transform infrared analysis of the nano-hydroxyapatite/chitosan (n-HA-CS) composite membranes prepared using solvent casting and evaporation methods. In addition to bands shift observations, the hydroxyl bands of n-HA disappeared after compounding. The authors reported that the disappearance of the hydroxyl band (at around 3570 cm<sup>-1</sup>) of n-HA and the movement of the CS polar groups was attributed to the formation of hydrogen bonds (Fig. 8a) between the hydroxyl groups on the surface of n-HA and the plentiful hydroxyl and amino functional groups of the polymer. On the other hand, it was shown that the amino groups (-NH<sub>2</sub>) of the CS polymer could participate in specific interactions with divalent ions like Hg<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Ni<sup>2+</sup>, Cd<sup>2+</sup>, Ca<sup>2+</sup>, and Co<sup>2+</sup> [133]. The Ca(II) ions, located on the terminated surface of n-HA apatite, have a coordination number of 7 and are tightly held in the HA structure. So, a coordination bond (Fig. 8b) might be formed

between the Ca(II) of n-HA and  $\text{-NH}_2$  of the CS polymer. Such a mechanism was reported in several studies [19,131]. Zima et al. [19] showed that the reaction between the Ca ions of the HA apatite and the amino groups of the chitosan (CS) polymer could take place within the HA-CS composite matrix. It was also suggested that the chitosan/calcium complexes formed can interact with phosphate ions of the apatite via electrostatic interactions, leading to the formation of a hybrid HA-CS composite.



**Fig. 8.** Schematic representation of possible chemical interaction between hydroxyapatite and chitosan: (a) Hydrogen bonding (----hydrogen bonds) between the OH group of hydroxyapatite and the  $\text{-OH}$  and  $\text{-NH}_2$  functional groups of chitosan, (b) Coordination bond between  $\text{Ca(II)}$  of hydroxyapatite and  $\text{-NH}_2$  of chitosan.

## **7. Physicochemical and biological properties of hydroxyapatite-chitosan composite**

In recent years, besides the development of new manufacturing technologies of HA and CS composites, the paradigm has been shifted toward the physicochemical and biological properties by emphasizing the improvement of the biological interactions of HA-CS composites scaffolds with bone tissues and cells as well as their use in bone tissue engineering. Mechanical performances and biological responses of these biocomposites are the driving forces in the translational applications and their commercialization for biomedical uses (Table 3).



**Table 3.** A summary of preparation methods and properties of hydroxyapatite-chitosan composite.

Composite	Preparation Method	Result	Application	Ref
<p>HA-CS (CS %: 3.44)</p>	<p>Co-precipitation</p>	<ul style="list-style-type: none"> <li>- Good degradability (in PBS)</li> <li>- Good integration and distribution of HA in the chitosan matrix.</li> <li>- Compressive modulus (<math>9.29 \pm 0.8</math> MPa) was significantly higher than chitosan alone (<math>3.26 \pm 2.5</math> MPa).</li> <li>- HA-CS composite provides good cell attachment and proliferation compared to CS.</li> </ul>	<p>Bone regeneration</p>	<p>[134]</p>
<p>HA-CS (CS %: 1 - 5)</p>	<p>Solid-liquid / freeze-drying</p>	<ul style="list-style-type: none"> <li>- Improved compressive strength with the increase in the amount and the molecular weight of the polymer enhanced the composite.</li> <li>- Increasing the solid to liquid ratio from 0.5 to 1 increased the composite compressive strength up to <math>2.40 \pm 0.34</math> MPa.</li> <li>- Good cohesion and mechanical stability.</li> </ul>	<p>Bone fracture repair</p>	<p>[17]</p>

HA-CS (CS %: 10 - 30)	Wet conventional synthesis	<ul style="list-style-type: none"> <li>- Good cytotoxicity <i>in vitro</i> (cell adhesion and growth)</li> <li>- Higher thermal stability compared to pure CS.</li> </ul>	Tissue engineering	[135]
HA-CS (CS %: 70 - 90)	Freeze-drying	<ul style="list-style-type: none"> <li>- High porosity and excellent swelling ability.</li> <li>- Higher compressive modulus compared to CS.</li> <li>- Improved cell attachment, biocompatibility, and osteogenic differentiation compared with pure CS scaffolds.</li> </ul>	Bone tissue engineering	[95]
HA-CS (CS %: 17 - 23)	Co-precipitation	<ul style="list-style-type: none"> <li>- Increased compressive strength (from 24,5 to 32,1 MPa) with the increase in the CS content from 17 to 23 %.</li> <li>- High compressive strength compared to pure HA (~ 2 MPa).</li> <li>- Good chemical stability in simulated body fluid (<i>in vitro</i>).</li> </ul>	Bone substitute	[19]
HA-CS (CS %: 20 - 70)	Co-precipitation	<ul style="list-style-type: none"> <li>- Improved mechanical strength ( 67 - 120 MPa).</li> <li>- Maximum value of the compressive strength was about 120 MPa for HA-CS containing 30 wt% of CS.</li> <li>- Excellent biodegradability et bioactivity in contact with SBF (<i>in vitro</i>).</li> </ul>	Bone substitute materials	[136]

<p>HA-CS (CS %: 99.5 - 98)</p>	<p>Freeze-drying</p>	<ul style="list-style-type: none"> <li>- High compression modulus compared to pure CS.</li> <li>- Decreased water uptake ability with the increase in the amount of HA.</li> <li>- Water retention ability similar to pure chitosan scaffold.</li> <li>- Good biological response of the nanocomposite scaffolds toward pre-osteoblasts (MC 3T3-E1).</li> <li>- Improved cell attachment and higher proliferation than chitosan scaffold.</li> </ul>	<p>Bone tissue engineering</p>	<p>[22]</p>
<p>HA-CS (CS %: 5 and 10)</p>	<p>Co-precipitation</p>	<ul style="list-style-type: none"> <li>- Higher resorption rate of the composites compared to pure HA.</li> <li>- Controlled solubility of HA by optimizing the content of chitosan into the composite matrix.</li> </ul>	<p>Bioresorbable bone substitute</p>	<p>[96]</p>
<p>HA-CS (CS %: 70 - 95)</p>	<p>Casting/solvent evaporation</p>	<ul style="list-style-type: none"> <li>- Excellent cytocompatibility, sufficient water adsorption, and tensile strength.</li> </ul>	<p>Tissue engineering</p>	<p>[98]</p>

HA-CS (CS %: 62.5)	Lyophilization - <i>in situ</i> hydration and simple blending	<ul style="list-style-type: none"> <li>- Good compressive strength compared to pure CS. Composites are prepared by lyophilization.</li> <li>- <i>In situ</i> hydration revealed excellent compressive strength and biological properties compared to simple blending</li> </ul>	Bone tissue engineering	[48]
HA-CS (CS %: 50)	Freeze-drying	<ul style="list-style-type: none"> <li>- Promote bone formation (<i>in vivo</i>)</li> <li>- Higher cell viability and proliferation of chitosan-nHA compared to Chitosan-mHA.</li> </ul>	Bone tissue regeneration	[38]
P-CS-CS-HA (CS %: 10, 20, 30, 40)	Co-precipitation method	<ul style="list-style-type: none"> <li>- The maximum compressive strength was measured at about 70.25 MPa</li> </ul>	bone tissue engineering	[137]
HA-CS (CS %: 25)	Co-precipitation	<ul style="list-style-type: none"> <li>- HA-CS scaffolds provide an effective space for new bone formation, proved by histological and histomorphometric result</li> </ul>	Bone regeneration	[138]
HA-CS	<i>In situ</i> precipitation	<ul style="list-style-type: none"> <li>- The composite scaffolds revealed antibacterial activity against Gram-positive strains</li> <li>- Cell viability tests : no not cytotoxicity was noted</li> </ul>	Bone replacement materials	[139]

HA-CS	3D bioprinting	- Chitosan and HA-CS scaffolds exhibited good mechanical support and highly active cell platforms as compared to the alginate bioink.	Bone tissue engineering	[125]
HA-CS (CS %: 30)	3D printing (direct ink writing) and freeze-drying	- Biocompatible and cell adhesion. - Good and high levels of viability for 7 days of culturing.	Bone tissue engineering	[123]
HA-CS	3D printing (using Z-Corp, Z-510 system)	- Good mechanical properties : Compression strength of these specimens resulted in 16.32 MPa (s = 2.843), E Modulus 4.4 GPa (s=2.113) with a porosity of 37.1%.	Bone implantation and replacement	[128]
HA-CS (CS%: 97.5 - 90)	3D printing	- HA addition to into the CS-based scaffolds increased the porosity and pore size of the printed scaffolds - The scaffold samples showed biocompatible properties and did not have toxicity toward rat bone marrow mesenchymal stem cells. - No considerable toxicity on MSCs was observed in 3D-printed scaffolds.	Personalized craniofacial bone tissue engineering	[126]

HA-CS	Direct Ink writing	<ul style="list-style-type: none"><li>- The modulus as well as the porosity of the 3D scaffolds, were proved to be within the respective value ranges of natural cancellous bone.</li><li>- Interconnected porous network</li><li>- Biocompatibility property</li></ul>	Regeneration of damaged and/or defected tissue	[127]
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Ying et al. [95] demonstrated a slight decrease in the porosity of the HA-CS composite scaffolds from  $83.39 \pm 1.01$  % to  $79.48 \pm 1.09$  % when raising the HA content from 10 to 30 %. The highest porosity of about  $86.96 \pm 0.83$ % was noted for pure CS scaffold. This decrease in the composite porosity was attributed to an absolute collapse of the composite pores. Nevertheless, the obtained values are enough to facilitate cell adhesion and proliferation. Elhendawi et al. [140] found that the temperature synthesis has a significant effect on the crystal structure and size of the HA-CS nanocomposites particles prepared using *in situ* co-precipitation technique. A decrease in the processing temperature (from 60 to 10 °C) led to a decrease in the crystallinity and the growth of HA, as confirmed by X-ray diffraction (XRD) and transmission electron microscopy (TEM). Moreover, the Ca/P measured for the composite precipitated at high temperature (60 °C) was higher than the solids obtained at lower temperatures (37 and -10 °C). A series of cross-linking HA-CS membranes made with various HA amounts (0, 5, 10, 15, 20 and 30 wt%) were fabricated through a new casting/solvent evaporation technique and investigated for bone tissue engineering applications [98]. The property of the composite membrane was greatly affected by the HA content. For higher HA content of more than 10 wt%, the separation of HA from the matrix membrane was visible by SEM analysis, resulting in a discontinuous structure. Escobar-Sierra et al. [23] investigated the effect of the preparation method on the HA-CS scaffolds morphology. The HA-CS composite obtained *in situ* has a 3D matrix with a highly porous structure and an interconnection between the pores. Furthermore, the composite porosity raises with the increase of the HA content. It was also reported that the HA addition into the chitosan-based scaffolds improved the porosity and pore size of the printed biomaterial using extrusion-based 3D printing [126].

Compressive strength is usually the imperative criterion when choosing bone substitutes for tissue engineering. Ait Said et al. [20], performed tests on cylindrical HA-CS composites formulated using the solid-liquid mixing method, showing that the addition of CS polymer (1 -

5 wt%) improved the compressive strength of the composite matrix (from  $1.11 \pm 0.22$  to  $3.60 \pm 0.72$  MPa). According to Zima et al. [19], using 17 wt% of CS resulted in about 12-fold increase in the composite compressive strength as compared to pure HA, while about 16-fold increase in the value of this property was observed in the presence of 23 wt% of the polymer. Similar findings were observed in the case of cylindrical HA-CS prepared via *in situ* precipitation method [136]. The authors proved that the compressive strength of the obtained composites raised from 102 to 120 MPa for HA-CS ratios ranging from 20/80 to 30/70, respectively. However, a decrease in the compressive strength of the composite from 120 to 67 MPa occurred when the HA-CS ratio increased from 30/70 to 70/30 wt% [98]. The increase in the mechanical strength of the composites with low polymer content (% of CS  $\leq$  30) was explained by different factors, like decreased porosity and strong interfacial interaction between amino functional groups of the CS and the calcium ions and/or negative phosphate species of the mineral phase. Other parameters can also lead to the improved mechanical response of these composites, like particle size and particle size distribution within the HA-CS matrix, good distribution of HA in chitosan, and the mechanical properties of the polymeric phase itself. The effect of the CS molecular weight was also reported for HA-CS formulated using solid-liquid mixing at a solid/ liquid ratio of 0.5 [17]. The result revealed that the compressive strength of the HA-CS<sub>M</sub>5% (5 % of CS) composite containing the polymer with medium molecular weight ( $0.3 \pm 0.06$  MPa) was four times higher than that of the HA-CS<sub>L</sub>5% (5 % of CS) containing the CS with low molecular weight ( $0.08 \pm 0.016$  MPa). The phosphorylated chitosan-chitosan-hydroxyapatite (PCS-CS-HA) composites with different PCS/CS/HA ratios (40/40/20, 30/30/40, 20/20/60, 10/10/80) were prepared by co-precipitation technique, and their compressive strength was evaluated. The results showed that the maximum value of compressive strength measured was about 70.25 MPa corresponding to the PCS-CS-HA composite with a PCS/CS/HA ratio of 30/30/40. The decay observed in certain cases was



related to the used high amount of the organic phase that could not provide high strength due to its own properties.

Other mechanical parameters like Young's modulus and tensile strength were also evaluated on the HA-CS composite scaffolds [95,98]. Ying et al. [95] demonstrated that Young's modulus of the HA-CS composite scaffolds, prepared by the freeze-drying method, was higher in comparison with the CS biopolymer. The HA-CS containing 10 wt% of the HA showed the maximum Young's modulus of approximately  $37.26 \pm 1.00$  kPa (at 20 % strain), which is two times higher than the value of CS alone ( $15.96 \pm 0.35$  kPa). In contrast, this mechanical parameter diminished with an increase in the HA content (from 10 to 30 wt%) and reached the value of  $27.37 \pm 0.36$  kPa. To improve the mechanical performances of HA-based composites and polymer scaffolds, different crosslinking techniques are performed through a chemical, physical or enzymatic process using crosslinking agents like glutaraldehyde and genipin [123,141,142]. The tensile strength of the cross-linking HA-CS membranes produced via a casting/solvent evaporation method and using genipin cross-linking agent raised from 30 to 73 MPa when increasing the HA amount from 0 to 30 wt% [98].

Although many HA-polymer composites have been developed, some problems persist with their mechanical properties [22,23,143]. Thein-Han et al. [22] reported that the mechanical properties of the HA-CS composite scaffolds produced via freezing and lyophilization were inadequate for bone regeneration, despite the excellent biological responses of the system. The main reasons could be related to (i) the weak interfacial interactions, (ii) not homogenous distribution of the HA or CS within the structure of the composite and (iii) poor attachment of the components.

The crucial parameter for scaffolds designed for bone engineering applications is their porosity and toughness. The mechanical strength of the biomaterial should mimic the construction of natural bone to meet the required standards. Thus, it is important to evaluate how the daily

humans activities affect the fatigue life of porous bioimplants. The new bone formation and bone regeneration process at the implant/natural bone interface needs to be understood and explored in greater detail. Adequate porosity within the implant structure is important for cell adhesion and better integration. Therefore, is also necessary to determine the optimal porosity level, as higher porosity could promote cell adhesion and facilitate bone growth, but it leads to deterioration in mechanical strength.

The swelling behavior is also another important property of the HA-CS composite scaffolds, which facilitates the infiltration of cells and biological molecules into the scaffolds matrix. The experiment can be performed by immersing the HA and CS composite in a phosphate buffer solution. Li et al. [98] reported that the swelling property of the CS polymer decreased from 1800 to 830 % when raising the HA content (from 0 to 30 wt%) in the composite matrix. Similar results were obtained by Tanase et al. [49] on the HA-CS composites, obtained through a biomimetic method. The authors demonstrated that the water absorption rate decreased with the decrease of HA content in the composite. This was explained by the decrease in the hydrogen bond between the water molecules and CS polymer macromolecules, as a result of chemical interactions between the apatite and the polymer, as confirmed by FTIR analysis. This could also be supported by the poor hydrophobicity of the apatite matrix and the decrease of the porosity of the composite in presence of the polymer. In fact, the absorption of water molecules into the composite takes place in two different processes: water retained in the pore volume of the materials matrix and chemical binding, especially with the amino ( $-NH_2$ ) and hydroxyl ( $-OH$ ) groups of chitosan [98,144,145]. The swelling property of the HA-CS composites scaffolds is an important property needed to enlarge the porosity and facilitate good cell adhesion and proliferation. However, this decrease in the water absorption rate within the composite matrix could diminish implant loosening in terms of mechanical property and stress to the surrounding tissue [146]. Researchers in the field of tissue engineering are also focusing

on combining the main physicochemical properties and biological features to produce effective biological substitutes, which are able of reproducing and maintaining normal functions in injured and diseased tissues [147].

The biodegradation ability of biomaterial scaffolds is critical for the long-term effectiveness of bone tissue-engineered implants [22,49]. The degradability of the CS polymer has been proved mainly in the presence of lysozyme, which exists in different body fluid solutions and human tissues. The HA biomaterial has good biocompatibility and osteoconductivity, but little biodegradability [148,149]. An *in vitro* biodegradation study of HA-CS nanocomposite scaffolds was performed in a phosphate-buffered saline (PBS) solution containing lysozyme [22]. The results showed that the scaffolds are degradable after 28 days. The degradation degree decreased when increasing the CS molecular weight (MW) and the HA content in the composite matrix. The mass losses of high MW CS and HA-CS composite were about 20 and 15 %, respectively, whereas medium MW CS and its composite scaffolds revealed losses of about 29 and 25 %, respectively. The low degradability of the composite scaffolds was attributed to both high crystallinity and a high degree of deacetylation (92.3%) of the CS polymer [22,49]. Another reason is that the HA crystals have a strong bonding with the polymer fibers which makes it difficult to be destroyed by the lysozyme. Earlier studies have reported that the degradation process in the lysozyme depends on the deacetylation degree of chitosan [150,151]. The molecular weight (MW), degree of deacetylation, and degradation rate of the polymeric phase are important parameters to consider in developing biocomposite materials for bone tissue engineering applications.

Involving functional biomaterials to regulate stem cells differentiation has attracted great attention in the biomedical field, particularly in bone repair and tissue engineering. Mesenchymal stem cells (MSCs) are potential cells source for bone tissue engineering. They are able to differentiate into a specific lineage like osteoblast, chondrocytes, and adipocytes

[90,152]. The capacity of nanofibrous HA-CS scaffolds to induce the differentiation of murine mesenchymal stem cells (mMSCs) into the osteogenic lineage was investigated [153]. The scaffolds supported better adhesion and proliferation of the mMSCs cells, and significantly up-regulated osteogenic genes such as collagen I, osteocalcin, runt-related transcription factor 2, and alkaline phosphatase, in the absence of the osteogenic environment. *In vivo* study performed by Zhang et al. [25] showed that bone regeneration on HA-CS biocomposite scaffolds was higher than pure CS scaffolds after the implantation into segmental bone defects of rabbits. In this research, critical size defects (6 mm Ø x 10 mm H) were created for 43 adults New Zealand white rabbits in the left femoral condyles. The defects were repaired separately using HA-CS composite, pure chitosan, or left empty. The results showed that the segmental bone defect repaired with the HA-CS composite was completely healed after 12 weeks, whereas the defect was still visible in the case of the chitosan-group.

The *in vitro* cytotoxicity study performed by Li et al. [98] revealed that the prepared HA-CS crosslinking membranes have non-cytotoxicity toward L929 cells and can serve as support for bone repair and tissue engineering applications. An *in vivo* study performed on biocomposites based on natural HA and chitosan showed that these materials possess excellent hard tissue biocompatibility and osteoconductivity, which is desirable for artificial orthopedic implants and frame biomaterials of tissue engineering [154]. The effect of the preparation method on the biocompatibility of HA-CS composite was studied [23,48]. Li et al. [48] reported that the HA-CS scaffolds obtained by chemical precipitation showed good biocompatibility, confirmed by better cell proliferation, compared to those obtained via simple blending. Escobar-Sierra et al. [23] demonstrated that the scaffolds produced by *in situ* precipitation allow good cell growth compared to those obtained using the mixing process. These results were explained by the good morphology (porous structure and interconnected pores) of the *in situ* precipitated composites. The crucial role of porosity on biological properties was highlighted in several studies.

Homogenous chitosan and HA composite scaffolds were prepared through wet chemical method coupled with lyophilization [93]. The obtained spongy composite scaffolds showed good biocompatibility in comparison to the chitosan scaffolds. Moreover, the composites promote the cells proliferation and growth due to its higher porosity compared to the polymer. The effect of HA particle size on biocompatibility and cell proliferation was *in vivo* investigated on HA-CS composite scaffolds with preosteoblast (MC3T3-E1) cells [38]. The results showed that the total volume, bone volume, and surface, trabecular number, trabecular thickness, trabecular separation were higher in HA-CS prepared with HA nano particles than in composite scaffolds with HA micro particles. These results were attributed to the presence and good distribution of HA particles within the HA-CS composite. The biocompatibility tests of the 3D printed HA-Chitosan scaffolds, using direct ink writing and freeze-drying process, were assessed using MG63 human osteosarcoma cells [123]. The results revealed that the composite constituted a cell-friendly environment, promotes the adhesion of cells onto the scaffolds surface, and improves cell proliferation with high levels of viability for 7 days of culturing. Chitosan-HA 3D printed composite scaffolds were investigated by Demirtaş et al. [125] for bone tissue engineering applications. The obtained results showed that the cells within the chitosan-HA and chitosan hydrogels had mineralized and differentiated osteogenically after 21 days of culture. Moreover, a comparative study has shown that both preprinted chitosan-HA and chitosan hydrogels yielded highly active cell platforms as compared to the alginate bioink. HA-CS scaffold samples with different ratios HA contents (2.5, 5, and 10%) were incorporated within the CS hydrogels structure through extrusion-based 3D printing, and freeze-drying to enhance the biological characteristics of the scaffolds. The results showed that the developed composites were biocompatible and did not undergo any toxicity against rat bone marrow mesenchymal stem cells. Moreover, the products containing 5% w/w HA revealed significant improvement in cell viability as compared to the control [126].

## 8. Doped-hydroxyapatite-chitosan composites

The relevance of HA as a bone repair is raised from the fact that the bone mineral is based on a poorly crystalline carbonate apatite and other substituent analogs of geological apatite [101]. Different substituents, both anions ( $\text{CO}_3^{2-}$ ,  $\text{F}^-$ ,  $\text{HPO}_4^{2-}$ ,  $\text{H}_2\text{PO}_4^-$ ) and cations ( $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{Fe}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Pb}^{2+}$ ,  $\text{Sr}^{2+}$ , ...), are found on the surface and within the lattice of the biological apatite crystals [132]. Great attention has been paid to ionic substitutions in the HA structure to widen the range of biomedical applications [107,155,156]. It can be used for replacing and repairing damaged and diseased parts of musculoskeletal systems.

Doped-HA-CS composites were also extensively investigated in order to further enhance the physicochemical and biological properties of the HA matrix [90,94,157,158]. The principal results are summarized in Table 4. Biocomposite scaffolds of Ag-HA-CS were developed by Saravanan et al. [159] through a freeze-drying technique. In this study, silver ions were introduced in a controlled amount by a reduction process mediated via the chitosan functional groups. The antibacterial test of the prepared composite revealed an antibacterial activity against Gram-positive and Gram-negative microorganisms. The scaffolds were also found to be non-toxic toward rat osteoprogenitor cells and human osteosarcoma cell lines. Silver-magnesium-hydroxyapatite/chitosan (Ag-Mg-HA-CS) nano-composites were produced by microwave-assisted chemical precipitation method [160]. Co-doping using  $\text{Ag}^+$  and  $\text{Mg}^{2+}$  was performed simultaneously within the HA structure resulting in the formula:  $(\text{Mg}_x\text{Ag}_y\text{Ca}_{(10-x-y)})(\text{PO}_4)_6(\text{OH})_2$ ; where  $0 \leq (x + y) \leq 1$ ). The obtained composites showed a surface morphology similar to that of natural bone.  $\text{Ag}^+$  dopant provided an anti-bacterial effect to the composite matrix and doping by  $\text{Mg}^{2+}$  led to an increase in the mechanical properties of the co-substituted HA biocomposites. The mechanical testing revealed that the maximum compressive strength of the developed biocomposite was about 15 MPa for the sample at zero  $\text{Ag}^+$ , and 0.8 of  $\text{Mg}^{2+}$ . Tripathi et al. [157] showed that the addition of copper-zinc (Cu-Zn) alloy nanoparticles in the

CS-HA nanocomposite, prepared by freeze-drying, significantly improved the swelling, protein adsorption, and the antibacterial activity of Cu-ZnHA-CS scaffolds. Afifi et al. [161] synthesized Mg-Sr doped-HA-CS using casting technique for orthopedic applications. The study revealed that Mg(II) induced high resistance to fracture and increased compressive strength (from  $7.17 \pm 1.1$  to  $15.1 \pm 1.5$  MPa). The prepared composite also exhibited good biocompatibility and high resistance for degradation in SBF medium. Strontium doped-HA-CS (SrHA-CS) nanohybrid scaffolds were successfully fabricated by Lei et al. [94] using a freeze-drying process, and the obtained nanocomposites displayed excellent cytocompatibility, acceptable adhesion and proliferation of human bone marrow mesenchymal stem cells. Furthermore, the alkaline phosphatase activity, extracellular matrix mineralization, and the osteogenic-related collagen type I and alkaline phosphatase expression levels were enhanced upon the  $\text{Sr}^{2+}$  ions released from the SrHA-CS scaffolds. It was reported that doping with strontium has attracted great attention from researchers due to its ability to inhibit bone resorption and positive impact on bone formation in osteoporotic patients [15]. Sr-Se-Zn-Mg-doped HA-CS was developed by Ressler et al. [27] using selective laser melting coupled with impregnation and freeze-gelation method for artificial bone graft substitutes. The resulted composite showed and improved mechanical properties, good cell attachment and homogeneous proliferation on the surface of the composite scaffold. Bi-SrHA-CS composite was developed by Zhipeng et al. [30] using precipitation method and freeze-drying. The results revealed that Bi-SrHA-CS composites exhibited good anti-tumor, anti-infective and osteogenic properties compared to SrHA-CS composites with improved niobium content. The authors reported that Bi crucial role in increasing the antibacterial, osteogenesis and antitumor activities of the composite.

**Table 4.** Main studies performed on doped-hydroxyapatite-chitosan composite.

<b>Composite</b>	<b>Preparation method</b>	<b>Results</b>	<b>Application</b>	<b>Ref</b>
Mg and Ag doped HA-CS	Microwave-assisted co-precipitation	<ul style="list-style-type: none"><li>- Surface morphology similar to that of natural bone.</li><li>- Ag<sup>+</sup> provides an anti-bacterial activity to composite</li><li>- Mg<sup>2+</sup> dopant improved the mechanical properties.</li></ul>	Bone tissue engineering	[160]
AgHA-CS	Freeze drying	<ul style="list-style-type: none"><li>- Antibacterial activity against Gram-positive and Gram-negative bacteria.</li><li>- Non-toxic to rat osteoprogenitor cells and human osteosarcoma cell line.</li></ul>	Bone tissue engineering	[159]
AgHA-CS	Lyophilization	<ul style="list-style-type: none"><li>- HA-CS scaffolds induced bacteriostasis against E. coli with an inhibition zone (15 mm) and high bactericidal rate (&gt; 99 %).</li><li>- Cell proliferation, morphology, and alkaline phosphatase activity are comparable to the control samples.</li></ul>	Bone tissue engineering	[162]



CuHA-CS	Microwave-hydrothermal	<ul style="list-style-type: none"> <li>- High degrees of porosity.</li> <li>- Sustained release of Cu ions.</li> <li>- CuHA-CS improved the osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells (rBMSCs).</li> <li>- Induce the migration and tube formation of EA.hy926 cells.</li> <li>- Promote bone regeneration in rats with critical size defects</li> </ul>	Drug delivery and bone regeneration	[163]
LaHA-CS	Precipitation and freeze drying	<ul style="list-style-type: none"> <li>- Induce the polarization of macrophage into the anti-inflammatory phenotype (<i>in vitro</i>)</li> <li>- LaHA-CS composite promoted the new bone regeneration</li> </ul>	Bone regeneration	[164]
MgHA-CS	Co-precipitation and magnetron sputtering technique	<ul style="list-style-type: none"> <li>- Physicochemical characterization : the apatite structure was not affected upon the ionizing radiation in the composite coatings</li> </ul>	Coating of orthopedic prosthesis	[165]
MgHA-CS	Spin-coating technique	<ul style="list-style-type: none"> <li>- No toxicity was observed toward osteoblast and HCT-8 cell lines <i>in vitro</i></li> <li>- Good antifungal properties against <i>Candida albicans</i> ATCC 10231</li> </ul>	Coating implants (orthopedics or dentistry)	[166]

MgHA-CS	In situ precipitation	<ul style="list-style-type: none"> <li>- Mg, Sr and Si affected the growth of the HA crystallites.</li> <li>- <i>In vitro</i> cell culture tests showed that the Mg-CS-HA, ZnHA-CS, SiHA-CS, and SrHA-CS had a higher proliferation than CS-HA.</li> <li>- The proliferative capacity of the cells cultured is in the following order: SrHA-CS- &gt; SiHA-CS &gt; ZnHA-CS &gt; MgHA-CS &gt; HA-CS.</li> </ul>	Bone tissue engineering	[167]
ZnHA-CS				
SrHA-CS				
SiHA-CS				
SrHA-CS nanohybrid	Freeze-drying	<ul style="list-style-type: none"> <li>- Sr-HA-CS and HA-CS showed better cytocompatibility with the acceptable adhesion and proliferation of human bone marrow mesenchymal stem cells.</li> <li>- Sr<sup>2+</sup> ions released from the SrHA-CS scaffolds improved the alkaline phosphatase activity, extracellular matrix mineralization, and osteogenic-related COL-I and ALP expression levels.</li> </ul>	Bone tissue engineering	[94]
SrHA-CS	Precipitation	<ul style="list-style-type: none"> <li>- High biocompatibility and osteoconductivity.</li> <li>- Enhanced <i>in vitro</i> drug release behavior and scarce cell toxicity. Great potential for spinal cord repair.</li> </ul>	Spinal cord repair	[168]

YbHA-CS	Precipitation	<ul style="list-style-type: none"> <li>-Enhanced osteogenic and angiogenic abilities</li> <li>-Fast formation of blood vessels in bone defects</li> <li>-Enhanced osteogenic differentiation of rBMSCs and in vivo bone tissue regeneration</li> <li>-Most of the defects were filled with new bone tissues after 12 weeks of operation</li> </ul>	Bone defect healing	[169]
ZnHA-CS	Wet mixing	<ul style="list-style-type: none"> <li>- The addition of chitosan improved the compressive strength and the modulus elasticity.</li> <li>- Non-toxic against BHK-21 cells.</li> </ul>	Bone substitute	[170]
ZnHA-CS	<i>In situ</i> precipitation and vacuum deposition method	<ul style="list-style-type: none"> <li>- Good antifungal activity</li> <li>- Improved bioactivity and biocompatibility</li> </ul>	Bioimplant	[171]
Mg/ SrHA-CS	Casting Technique	<ul style="list-style-type: none"> <li>-Mg(II) induced high resistance to fracture and increased compressive strength (from <math>7.17 \pm 1.1</math> to <math>15.1 \pm 1.5</math> MPa)</li> <li>-Good biocompatibility</li> <li>-High resistance for degradation in SBF medium.</li> </ul>	Orthopedic applications	[161]

Bi/Sr-HA-CS	Precipitation	-Bi-SrHA-CS composites revealed good anti-tumor, anti-infective and osteogenic property compared to SrHA-CS composites	Treatment of osteosarcoma, bacterial and bone defects	[30]
Sr-Se-Zn-MgHA-CS (CS %: 70)	Selective laser melting coupled with impregnation and freeze-gelation method	-Cell attachment and homogeneous proliferation on composite scaffold -Improved mechanical properties of CHT/sHAp hydrogel have been.	Artificial bone graft substitutes	[27]

## 9. Hydroxyapatite-chitosan composite as a drug delivery system

The treatment of bone infections or diseases is a complex and long process. The conventional treatment includes long-term systemic use of drugs and expensive surgical removal of the dead or infected bone, and adjacent soft tissue [172,173]. The long-term use of drug molecules, particularly in high doses, has been proved to cause several side effects due to the over suppression of bone resorption. The design of local drug delivery carriers can significantly enhance the treatment of bone infections and diseases [14,15,20,172,174]. Sufficient drug concentrations can be achieved at target sites and at the perfect time without any systemic toxicity. According to the literature, great attention has been paid to antibiotics molecules, this is due to their wide use: either as prophylactics to avoid bone infections appearing upon surgical interventions, or generally to treat bone-associated infections. For prophylaxis, the kinetic drug release from the carrier matrix must be sufficient to achieve concentrations higher than the drug minimum inhibitory concentration (MIC) to avoid bacterial resistance.

Many studies have been performed on HA-CS composites as drug delivery systems (Table 5). These drug-loaded-HA-CS composites can be developed into different shapes, functions, and structures, to answer several requirements for the treatment of bone diseases and infections. The drug-HA-CS composites often demonstrate good biological properties and drug-release ability. Drug molecules like antibiotics, anticancer, anti-inflammatory, and growth agents could be incorporated into the hydroxyapatite-chitosan matrix [175–177]. The presence of amino groups in the CS backbone confers a unique polycationic character to its structure that ensures the encapsulation or adsorption of negatively charged organic molecules [84,178]. Simultaneously, the inherent bioadhesion, biodegradability, and modifiability of this polysaccharide allow its use as a biomaterial component to produce efficient drug delivery carriers with controlled drug release and enhanced bioavailability. On the other hand, HAs of various surface properties and morphologies have been investigated as drug support for the

local delivery of a variety of biologically active molecules, due to their controlled size, highly active surface, structural advantages, ease of modification, and nontoxicity. Most of the research performed on the functionalization of CaPs as a drug delivery vehicle was performed on HA apatite. HA biomaterials were widely used to deliver antibiotics molecules directly to hard tissues and also other drugs like anti-osteoporotics, anti-cancer substances, anti-inflammatory, vitamins, proteins growth factors, and hormones [12,14,174]. Very few studies have been conducted on other CaPs ceramics, namely, OCP and  $\beta$ -TCP [101]. The characteristics of HA offer the possibility of using composite based on HA and CS not only as a biocompatible bone repair but also as drug delivery systems.

The effectiveness of any local drug delivery system depends on various parameters like microstructure (e.g., specific surface area, permeability, and porosity), degradation of the carrier matrix, preparation method, drug solubility, and functional groups [12,179]. The preparation method is one of the important parameters that determine the release kinetics and the drug distribution within the composite matrix. The drug can be incorporated either in a powder phase or dissolved in the liquid phase of the CaP matrix [15]. In the liquid phase, the antibiotic molecules could be adsorbed onto the surface of the drug carrier during the impregnation and free through the diffusion mechanism.

In general, drug release from HA-based drug delivery carrier revealed a significant initial rapid release during the first 24 h, in some cases eluting even the totality of the drug molecules. To overcome this problem, the development of HA-CS has been considered as an appealing approach to delay drug release, due to the high viscosity, complexation property and excellent degradation rate of CS polymer [20,180–182]. Uskoković et al. [181] attempted to overcome the burst liberation of clindamycin antibiotic from HA, by coating the apatite surface with the chitosan polymer. The results demonstrated the presence of the polymer mitigated the fast release of the antibiotic over the first 24 h. Thus, only 15 % of the antibiotic was eluted from

the composite on the first day, as opposed to 60 % from clindamycin-loaded HA. On the other hand, Tıǧlı et al. [183] combined the chitosan with HA beads (40  $\mu\text{m}$ ) in order to prolong the release of basic fibroblast growth factor (bFGF). The bFGF loading into the composite scaffolds was conducted via the embedding technique. Sustained drug release (up to 120 h) was achieved for composite scaffolds manufactured in the presence of HA beads, compared to bFGF-loaded chitosan (total release after 20 h). This behavior was explained by the electrostatic interactions between the bFGF molecules and the HA particles. However, the authors demonstrate that the drug release is described by an anomalous transport, attesting to the erosion and swelling phenomenon of the scaffold. HA-CS microspheres were prepared via microfabrication technology coupled to emulsification technic, and loaded with the polyethylene imine/bone morphogenetic protein 2 plasmid (PEI-pBMP2) complexes. The maximum loading capacity of PEI-pBMP2 complexes was about  $59.79\% \pm 1.85\%$ . Moreover, the study revealed that the release process of the plasmids occurred in a slow and controlled manner for up to 21 days [184].

The effect of the HA content on the drug release from porous HA-CS scaffolds loaded with tetracycline antibiotic, fabricated through precipitation and freeze-drying process, was investigated in PBS buffer solution [180]. The authors concluded that increasing the HA content decreased the release rate and allowed a more gradual elution of the antibacterial agent for up to 120 h. However, the *in vitro* drug release test from the porous scaffolds showed a rapid antibiotic release occurred within the first 2 h. This burst release was avoided by coating the composite scaffolds with a dense and nondrug-loaded layer. The antibiotic release process was prolonged over 240 h.

Reves et al. [175] used the lyophilization process to enhance the drug delivery ability of chitosan-HA microspheres loaded separately with alkaline phosphatase (ALP), bone morphogenetic protein-2 (BMP-2) growth factors, and amikacin antibiotic for orthopedic

applications. The results showed that the ALP and BMP-loading profiles were improved using lyophilization, which is due to the expanded surface area, additional porosity, and roughened surface texture of the composite scaffolds. However, there was no significant difference in the amikacin amount loaded between the non-lyophilized and lyophilized microspheres. These results were explained by the smaller molecular weight of the amikacin antibiotic in comparison with the ALP and BMP-2 proteins. A vancomycin-loaded-hydroxyapatite-chitosan was prepared by Zhang et al. [185] using a freeze-drying process. The release of vancomycin molecules was investigated using an SBF medium. The cumulative release profile showed a burst release of about 80 % during 10 h. The initial fast release was assigned to the weak antibiotic adsorption onto the composite surface. This fast period was followed by a slow release and approximately complete release after less than 24 h. The slow release during the rest of the time was explained by hydrogen bonding with the antibiotic carboxyl groups and the hydroxyl groups on the composite surface. The *in vivo* pharmacokinetics of vancomycin revealed that the therapeutic concentration of the antibiotic at the site of implantation was higher than the MIC, which was appropriate to ensure antimicrobial activity.

Our previous study performed on the hydroxyapatite-chitosan-ciprofloxacin composite formulated by a solid-liquid mixing method revealed a sustained and controlled drug release over 10 days of the experiment [20]. The drug release rate was controlled by varying the antibiotic content in the composite matrix. The HA-CS composite loaded with 9 % of the ciprofloxacin antibiotic revealed a significantly lower release rate (52 %) than that containing only 3 % of the antibiotic (release rate of 87%). It was also demonstrated that the release process was ruled by diffusion mechanism, and the antibiotic amounts released were at a therapeutic level for the treatment of microorganisms such as *Staphylococcus aureus*, and *Escherichia coli*. This study also revealed a rapid release process of the drug during the first 24h, attributed to the dissolution of the CIP molecules at the surface of the carrier and the release of the drug



molecules weakly adsorbed onto the surface of the composite scaffolds. Tablets of HA-CS loaded with ciprofloxacin can be prepared by solid-solid mixing [24]. In an animal study, ciprofloxacin incorporated HA-CS composite possesses a sustained antibiotic release *in vivo*. The released doses were above the MIC (0.25 - 2 µg/ml) of the antibiotic. Moreover, the release mechanism was ruled by the Fickian diffusion release process, implying a slower degradation of the composite matrix compared to their drug rate release. Minocycline loaded HA-CS composite with controlled release of the drug was investigated by Gao et al. [114]. A rapid release of the drug was noted during the second day of the experiment from the examined composite, while releasing a safe amount of minocycline in a sustained and controlled manner for up to 28 days. The antibacterials study revealed good antibacterial activity against *Staphylococcus aureus* and *Porphyromonas gingivalis* bacteria. Moreover, *in vivo* tests demonstrated that the drug-HA/CS material showed better effect of promoting periodontal bone formation. HA-CS composite fibers loaded with doxycycline were prepared via a crosslinking technic for maxillary bone regeneration [186]. The *in vitro* release study revealed a burst release of the drug during the first 6 h, then the drug was eluted in controlled maner over 72 h. A high drug release amount was noted for the compoaite formulation composed of 50 % HA. This was attributed to the physical adsorption of the antibiotic onto the HA surface, inducing a more sustained and controlled drug release. Although, the crosslinking solution in this study should be optimized to allow a milder condition and ensure an homogeneous desitribution of the polymer within the composite structure. This could be obtained by combining the CS biopolymer with other polymers.

The reactivity of HA surface has been explored to develop new HA-CS loaded with anticancer molecules for the treatment of bone cancer disease. Venkatesan et al. [177] envisaged HA-CS composite as potential support for the celecoxib drug. The precipitated celecoxib-loaded HA-CS nanocomposite showed high encapsulation efficiency and sustained release profile for 15

days (release rate of  $83.56 \pm 15.23$  %). The drug release from the composite nanoparticles was governed by the diffusion mechanism. The sustained release of the drug was explained by the low solubility and high viscosity of the polymer phase upon immersion in the release medium. The authors also suggest that the gradual degradation of the polymer and the creation of a porous structure within the composite matrix could facilitate slow drug release. HA-CS composite scaffolds were developed to be used as alternative drug delivery carriers of dexamethasone and basic fibroblast growth factor for periodontal bone regeneration applications [183]. The results showed that the developed scaffolds are efficient for the release of dexamethasone; however, the presence of the HA inorganic phase in the chitosan scaffold is necessary to provide the desired release rate of basic fibroblast growth factor.

The inherent osteoinductive and reactivity of the CS polymer have received immense attention and became one of the most appealing polysaccharides in developing local drug delivery carriers to treat bone infections and diseases. The promising anticancer activity of the celecoxib-loaded HA-CS scaffolds has been found [177]. The CS polymer plays an important role in supporting cell adhesion and proliferation, while the celecoxib drug shows an excellent anti-proliferation of colon cancer cells and significant inhibition of tumor growth *in vivo*. A hydroxyapatite/chitosan loaded with ciprofloxacin was investigated *in vivo* by Ren et al. [24]. The composite exhibited excellent antibacterial activity against *Staphylococcus aureus* bacteria, good cell (marrow mesenchymal stem) proliferation and adhesion, and provided a better bone repair capacity. Ait Said et al. [20] demonstrated that HA-CS composite loaded with ciprofloxacin antibiotic has excellent antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* pathogens, which cause osteomyelitis. Zhang et al. [185] reported that the hydroxyapatite-chitosan-vancomycin exhibited strong antibacterial activity *in vivo*. In this study, the number of methicillin-resistant *Staphylococcus aureus* (MRSA) on the vancomycin loaded-HA-CS after implantation was significantly less than on the composite without the

antibiotic. For example, the number of MRSA on composite loaded with vancomycin was  $(3.5 \pm 0.5) \times 10^3$  CFU after implantation for 7 days, whereas the test revealed  $(2.4 \pm 0.6) \times 10^6$  CFU for the composite without antibiotic. However, studies have reported that the antibacterial efficacy of hydroxyapatite-chitosan composites loaded with the antibiotic was lower than that of hydroxyapatite-antibiotic. Uskoković et al. [181] investigated the antibacterial activity of clindamycin loaded-hydroxyapatite-chitosan composite by measuring the MIC and using agar diffusion. Both experiments demonstrated that the composite had excellent antibacterial activity against *S. aureus*, but it was lower than the antibiotic-loaded HA. These results were explained by the rapid release of the antibiotic from HA support in absence of the chitosan.

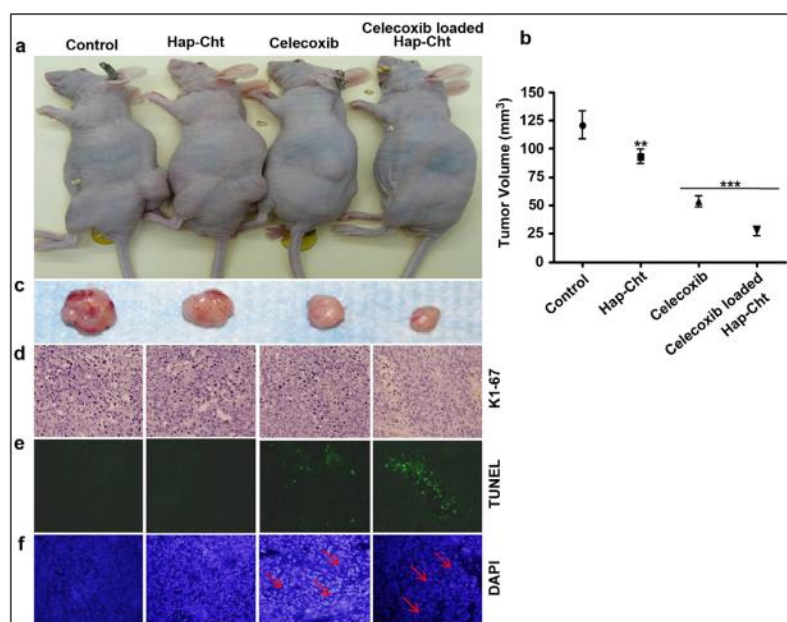
HA-CS composite scaffolds loaded with zoledronic acid were developed to be used in bone tumor therapy [187]. The prepared scaffolds exhibited excellent tumor inhibition activity against giant cell tumor of bone *in vitro* via inducing cells apoptosis as well as good antibacterial activity against *S. aureus* and *E. coli* bacteria. They exhibited good biocompatibility and osteoinductivity than pure HA-CS scaffolds toward human bone marrow mesenchymal stem cells.

In drug delivery applications, scaffolds with high porosity and specific surface area, controlled and good distribution of the pores size, and sufficient hardness are the most recommended to provide a sustained, controlled, and local release of the drug [14,20,188]. The utilization of composites based on HA and CS provides most of these properties. The *in vivo* drug release ability and antibacterial activity of mesoporous HA-CS composite scaffolds loaded with vancomycin (VCM) were investigated [185]. The number of viable methicillin-resistant *S. aureus* (MRSA) was significantly reduced after the implantation of VCM loaded-HA-CS composites. The developed composite scaffolds may be promising biomaterials used as drug storage and release vehicles for local treatment of bone infection as well as bone fracture repair. HA-CS composites are also used as drug-delivery systems for tumor treatments. The inhibition

of HCT 15 cells proliferation and tumor growth was evaluated *in vivo* in human colon mice xenografts models (Fig. 9) using celecoxib-loaded hydroxyapatite-chitosan (HA-CS) nanoparticles [177]. Venkatesan and coworkers observed that the celecoxib-loaded HA-CS nanoparticles exhibited higher tumor growth inhibition and low toxicity when compared to free celecoxib after injection via the tail vein with an equivalent concentration of 100 mg/kg of celecoxib drug (Fig. 9a). The tumor volume was smaller in the case of celecoxib-loaded HA-CS nanoparticles, followed by free celecoxib and HA-CS (Fig. 10b). The effect of drug-loaded nanoparticles on cell proliferation and apoptosis was also evaluated by Ki-67 (proliferation), dUTP nick end labeling (TUNEL) and 4'6-diamidino-2-phenylindole (DAPI) staining (apoptosis). Free celecoxib, HA-CS, and celecoxib-loaded HA-CS nanoparticles treated mice xenograft tumors revealed reduced expression of Ki-67 and raised TUNEL staining when compared to the untreated groups (Fig. 9d and e). This nanocomposite was suggested to be a promising delivery system for celecoxib to improve its efficacy in colon cancer chemotherapy. Doxorubicin-chitosan biocomposite coating on Ti alloy post HA coated has been successfully performed using a new technic by an electrochemical deposition approach for future clinical applications. A sustaining release of the drug over was noted. Besides, UV visible analysis and MTT tests showed an inhibition effect in the proliferation of tumor cells, which explained by because of the smaller drug particles for the Dox-HA-CS composited specimen. Although efforts to further improve the drug loading capacity is crucial to ensure the clinical efficacy [189].

An important issue that will influence the kinetics release of the drug is its distribution within the carrier matrix. Therefore, drug uptake process onto the surface of the composite should be studied before its application. However, up to now very investigations have been performed on the interaction between the composites particles and the drug molecules [190], it is therefore necessary to undertake further studies in this direction.

As previously mentioned, the drug molecules can be combined with the carrier surface either in the powder phase or dissolved in the liquid phase. When the carrier powder and drug solution are mixed, eventual dissolution of the solid particles could take place, resulting in a precipitation of new phases. The dissolved drug molecules are not expected to be impregnated inside the solid, or it would be in a small amount. Therefore, it is necessary to evaluate the stability of the composite structure after the addition of the drug.



**Fig. 9.** Human colon tumor xenografts in mice performed by injecting HCT 15 cells in the right flank of a nude mouse: (a) tumor-inhibiting effect of tail vein injections of celecoxib, HA-CS, and celecoxib-HA-CS composite on nude mice with HCT 15 xenografted carcinoma cells, (b) tumor volume and (c) image of excised tumors at the sacrifice time (30 days post-treatment). Tumors from the different treatment groups were immunohistochemically analyzed for Ki-67 expression and apoptosis (TUNEL and DAPI). (d) Tumor treatment with celecoxib reduced the number of Ki-67-positive cells, (e) raised the number of TUNEL-positive cells, and (f) nuclear fragmentation. Reprinted from Ref. [177] with permission.

**Table 5.** Main results on the drug release from hydroxyapatite-chitosan (HA-CS) composite.

Composition	Drug	Preparation method	Study	<i>In vitro</i> release	Other studies	Ref
<b>Antibiotics</b>						
HA-CS (CS : 5 %)	Ciprofloxacin (3 and 9 %)	Solid-liquid/freeze-drying	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Sustained release over 10 days (45 - 50 %).</li> <li>- Release rate during the first day: 25 %.</li> <li>- Drug release rate was antibiotic dose-dependent.</li> <li>- Fickian diffusion release mechanism.</li> </ul>	- Good antibacterial activity against <i>S. aureus</i> and <i>E. coli</i> pathogens.	[20]
HA-CS (CS : 5, 15 and 30 wt%)	Ciprofloxacin (3 and 9 %)	Solid-liquid method coupled and freeze-drying	<i>In vitro</i>	- Sustained and controlled CIP release for up to 10 days (52 - 87 %).	- Increased compressive strength 10-fold when compared to the HA-CS5 formulation (3.6 ± 0.7 MPa).	[190]

				<ul style="list-style-type: none"> <li>- Fast release during the first day (up to 32 %).</li> <li>- Drug release rate was antibiotic dose-dependent.</li> <li>- The release process is mainly governed by Fickian diffusion mechanism.</li> </ul>	<ul style="list-style-type: none"> <li>- Decreased from <math>36.8 \pm 8.5</math> MPa down to <math>20.5 \pm 4.7</math> MPa with the CIP addition (up to 9 wt%).</li> <li>- Antibacterial effect against <i>S. aureus</i> and <i>E. coli</i> bacteria.</li> </ul>	
HA-CS	Ciprofloxacin (10 %)	Solid mixing	<i>In vitro/ in vivo</i>	<ul style="list-style-type: none"> <li>- <i>In vivo</i> concentration of CIP above the MIC (0.25 - 2 µg/ml).</li> <li>- The antibiotic concentration released from the composite was higher than that from HA-CIP.</li> <li>- Fickian diffusion release mechanism.</li> </ul>	<ul style="list-style-type: none"> <li>- Excellent antibacterial activity against <i>S. aureus</i> bacteria.</li> <li>-HA-CIP exhibited high inhibition zone of than HA-CS-CIP composite.</li> <li>-Good cells (marrow mesenchymal stem) proliferation and adhesion.</li> <li>- Better bone repair capacity.</li> </ul>	[24]

HA coralline - CS (HA : 65 %, CS : 35%)	Gentamycin (47.5 %)	Encapsulation	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Fast <i>in vitro</i> release during the first day (~ 40 %).</li> <li>- Elution of about 84.9 % over 4 days in PBS.</li> </ul> <p>The release is governed by a zero-order model.</p>	-	[191]
HA-CS (CS %: 80 and 60)	Tetracycline hydrochloride (10 %)	<i>In situ</i> precipitation/ freeze-drying	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- About 30 % of the antibiotic was released after 2 h.</li> <li>- Release rate ranges between 93 - 86 % at 120 h.</li> </ul> <p>Diminution of the release rate with the increase of the HA content.</p>	-	[180]
HA-CS (CS %: 15 - 90)	Doxycycline	Crosslinking	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Burst release during the first 6 h</li> <li>- Controlled released from the composite fibers during 72 h</li> </ul>	-	[186]



HA-CS microspheres (CS %: 50)	Tetracycline hydrochloride	Adsorption	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Rapid release in PBS solution (within 3 h)</li> <li>- The release process was prolonged up to 68.8 % after 24 h in the presence of urchin-like nanofibers</li> </ul>	<ul style="list-style-type: none"> <li>- Good adsorption capacity (<math>26.2 \pm 7</math> - <math>62.9 \pm 0.4</math> %) of the antibiotic.</li> <li>- Antibacterial activity against <i>E. coli</i> and <i>S. aureus</i> bacteria</li> </ul>	[192]
HA-CS	Erythromycin	Encapsulation	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Burst release of the drug over the first 6<sup>th</sup> h</li> <li>- Sustained release during 96 h.</li> <li>- Higher drug release rate at pH 5.4 (47.79 %) compared to pH 7.4 (30.88 %).</li> </ul>	<ul style="list-style-type: none"> <li>- Drug encapsulation and loading capacity was about <math>69.28 \pm 0.77</math> % and <math>16.07 \pm 0.10</math> % respectively</li> <li>- Good inhibition of <i>B. cereus</i> and <i>S. entrica</i> bacteria</li> <li>- Hemolytic and CAM assays showed no toxicity</li> </ul>	[193]
HA-CS (CS %: 30)	Vancomycin (28.6 %)	Freeze-drying	<i>In vivo</i>	<ul style="list-style-type: none"> <li>- Burst release (80 %) within 10 h.</li> <li>- Complete drug release after 24 h.</li> </ul>	<ul style="list-style-type: none"> <li>- Significant diminution in the number of methicillin-resistant <i>S. aureus</i> after implantation</li> </ul>	[185]

				- Antibiotic concentration was higher than MIC at the site of implantation for 4 weeks ( <i>in vivo</i> ).		
HA-CS (CS %: 4.2)	Clindamycin (0.1 and 29 %)	Adsorption	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Drug release rate in the first 24 h was about 15 %.</li> <li>- Elution of 60 % of the antibiotic from pure HA during the first 24 h.</li> <li>- Sustained drug release kinetics throughout 21 days.</li> <li>- Slow drug release compared to clindamycin-HA</li> </ul>	<ul style="list-style-type: none"> <li>- Reduced antibacterial property against <i>S aureus</i> in presence of the polymer.</li> <li>- Diminished cell proliferation (osteoblastic) on HA-chitosan composites compared to HA.</li> </ul>	[181]
HA-CS (CS %: 20)	Minocycline	Co-precipitation	<i>In vitro/ in vivo</i>	<ul style="list-style-type: none"> <li>- Fast drug release during during the second day</li> <li>- Sustained release process of the drug during the</li> </ul>	<ul style="list-style-type: none"> <li>- Prolonged setting time with the drug addition</li> </ul>	[114]

				<ul style="list-style-type: none"> <li>- About 0.5 - 1 <math>\mu\text{g/day}</math> was released during the first month.</li> </ul>	<ul style="list-style-type: none"> <li>- Increased pore size and porosity and reduced compressive strength in presence of the drug</li> <li>- Promote the growth of osteoblasts cells compared with the control.</li> <li>- Better effect of promoting periodontal bone formation <i>in vivo</i></li> </ul>	
HA-CS	Amikacin	Adsorption	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Sustained drug release.</li> <li>- Lyophilized microspheres released more amikacin than those non-lyophilized.</li> <li>- Burst release process.</li> <li>- Extended elution profile by a supplemental chitosan coating.</li> </ul>	<ul style="list-style-type: none"> <li>- Lyophilization does not affect the cellular response of the composite.</li> </ul>	[175]

HA-CS	Gentamicin	Electrophoretic deposition process	<i>In vitro</i>	<ul style="list-style-type: none"> <li>-Burst release process in the first 48 h (~21% of gentamicin released)</li> <li>-Sustained release over 21 days (release rate up to 60 %)</li> </ul>	<ul style="list-style-type: none"> <li>-Noncytotoxic effect was observed against human and mice fibroblast cells (viability &gt;85%)</li> </ul>	[194]
<b>Anti-inflammatory</b>						
HA-CS	Ibuprofen	Precipitation	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Controlled release during 1500 min</li> </ul>	-	[176]
	Acetyl salicylic acid			<ul style="list-style-type: none"> <li>- Ibuprofen membranes present less release than acetylsalicylic acid ones.</li> </ul>		
HA-CS	Silymarin	Embedding	<i>In vitro</i>	-	<ul style="list-style-type: none"> <li>- Good anti-inflammatory activity.</li> <li>- Cytotoxicity test: minimal lethality against brine shrimps.</li> </ul>	[195]
HA-CS (60 %)	Resveratrol	water/oil emulsion	<i>In vitro/ In vivo</i>	<ul style="list-style-type: none"> <li>-Burst-release within the first 24 h.</li> </ul>	<ul style="list-style-type: none"> <li>- Anti-inflammatory activity</li> <li>- Promote osteo-differentiation.</li> </ul>	[190]

				- Sustained and controlled drug release (up to 120 h).	- <i>In vivo</i> test in osteoporotic rat femoral condyles revealed enhanced entochondrostosis and bone regeneration.	
HA-CS (CS %: 61.5 and 70.6)	Dex (9 - 23 %)	Freeze- drying/Embedding method	<i>In vitro</i>	- Sustained-release kinetics. - Complete release of Dex after a period of 2 to 5 days. - Dex release was controlled by Fickian.	-	[183]
<b>Anticancers</b>						
HA-CS (CS %: 20)	Celecoxib	Solid-liquid	<i>In vitro/ in vivo</i>	- About 13.78 % of the drug was eluted in the first 24 h. - Sustained release of the celecoxib (83.56 %) during 15 days. - Elution follows the diffusion mechanism.	- <i>In vitro</i> study showed anti-proliferation of colon cancer cells. - <i>In vivo</i> tests demonstrated significant inhibition of tumor growth.	[177]

SrHA-CS-	Norcantharidin	Coprecipitation and freeze-drying	<i>In vitro</i>	-	<ul style="list-style-type: none"> <li>- Inhibition in the growth of tumors.</li> <li>-Increased antitumor performance with the raise in the drug content.</li> <li>- Improve proliferation of osteoblasts</li> </ul>	[30]
HA-CS	Doxorubicin	Electro-deposition	- <i>In vitro</i>	<ul style="list-style-type: none"> <li>- Slower drug release for drug-HA-CS (up to 7.14 %) coating compared to drug-CS/Ti (up to 14.73 %) coating during the first day</li> <li>- Diffusion release mechanism</li> <li>- Sustained release over 28 day</li> </ul>	<ul style="list-style-type: none"> <li>- Inhibition in the proliferation of tumor cells</li> </ul>	[189]
<b>Growth factors</b>						
HA-CS	ALP	Adsorption	<i>In vitro</i>	- Sustained drug release.		[175]

	BMP-2			<p>-Lyophilized microspheres released more ALP than those non-lyophilized.</p> <p>-Burst release during the elution of BMP-2 drug.</p> <p>Extended elution profile by a supplemental chitosan coating.</p>	<p>-Lyophilization increased the ALP- and BMP-2-loading capacity of the composite microspheres.</p> <p>Lyophilization does not affect the cellular response of the composite.</p>	
HA-CS (CS %: 61.5 and 70.6)	bFGF (1,6 - 3.23 %)	Freeze- drying/Embedding method	<i>In vitro</i>	<p>- Sustained and complete release within 7 days.</p> <p>- Controlled release.</p> <p>bFGF release mechanism was controlled by anomalous diffusion.</p>	-	[183]
<b>Others</b>						

HA-CS (CS %: 25)	Human amelogenin	Embedding method	<i>In vitro/in vivo</i>	<ul style="list-style-type: none"> <li>- Burst initial hour release burst first day</li> <li>- Sustained release of the drug over 7 days</li> <li>Slow drug release from the composite formulation (67 %) compared to HA (89 %).</li> </ul>	<ul style="list-style-type: none"> <li>- Antibacterial activity against <i>Fusobacterium nucleatum</i> and <i>Porphyromonas gingivalis</i></li> <li>Induced the formation of cementum-like tissue <i>in vivo</i></li> </ul>	[196]
HA-CS	Metformin (antidiabetic)	Hydrothermal and immersion	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Rapid increase at the first 24 h of immersion in SBF solution.</li> <li>Sustained release over 20 days.</li> </ul>	<ul style="list-style-type: none"> <li>- HA-CS composite coating on AZ31 magnesium alloy enhances corrosion resistance.</li> <li>- Promote the expression level of osteogenic related genes.</li> <li>- Excellent biocompatibility.</li> </ul>	[197]
HA-CS (CS %: 40 – 75)	Tramadol	Impregnation	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Slight drug release during the first hours.</li> </ul>	<ul style="list-style-type: none"> <li>- Small pores and high specific surface.</li> </ul>	[198]



				- Sustained release over 300 h (100 % of release) PBS solution.	- The maximum mechanical strength was about to 23.9 MPa and 20.7 MPa in dry state and wet state, respectively.	
HA-CS	Zoledronic acid	Co-precipitation	<i>In vitro</i>	-	- The prepared CS/nHA/Zol scaffolds revealed excellent tumor inhibition. - Activity against giant cell tumor of bone. - Good biocompatibility and osteoinductivity than pure HA-CS scaffolds. - Good antibacterial activity against clinical pathogenic <i>S. aureus</i> and <i>E. coli</i> .	[187]

HA-CS	ATP	Adsorption	<i>In vitro</i>	<ul style="list-style-type: none"> <li>- Controlled drug release ability</li> <li>- Sustained release over 3 days</li> </ul>	<ul style="list-style-type: none"> <li>- Improved colloidal stability and controlled release ability</li> <li>- Better inhibition effect against the proliferation of the tumor cells compared to free ATP</li> <li>- No significant cytotoxic effect</li> <li>- High rate of apoptosis in the tumor cells</li> </ul>	[199]
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ALP: alkaline phosphatase; BMP-2: bone morphogenetic protein-2; bFGF: Basic fibroblast growth factor; CIP: ciprofloxacin; Dex: dexamethasone;

MIC: minimum inhibitory concentration; *S aureus*: *Staphylococcus aureus*; *Escherichia coli*: *E. Coli*. Adenosine 5'-triphosphate: ATP

## 10. Application as gene delivery carriers

Gene therapy is an emerging strategy for repairing and regenerating bone defects, with a particular focus on the use of exogenous deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) systems to regulate gene expression at the fracture site [200–202]. The inability of RNA and DNA to penetrate cell membranes and selectively deliver to the targeted tissues or cells within the body is a serious barrier to clinical translation. Moreover, their efficiency is frequently impeded by their vulnerability to degradative enzymes. Therefore, it is necessary to develop efficient delivery carriers with controlled, and sustainable delivery of genes for successful repression, silencing, and regulation of genes by DNA and RNA.

In 1996, the first scaffold-based gene-delivery carrier was developed for the treatment of musculoskeletal defects. Collagen scaffolds loaded with genes producing BMP-4 and human parathyroid hormone 1-34 fragment were implanted in rat femoral lesions and canine tibial defect [203,204]. The formation of new bone tissue and the bridging of significant segmental anomalies were observed. These scaffolds were also shown to be able to retain plasmid (p) DNA for more than 6 weeks and induce new bone formation in the case of canine tibial defect model.

Viral and non-viral vectors were designed for an effective gene transfer [201,202]. Viral strategies are an effective technic of gene transfer; but, safety issues like insertional mutagenesis restrict their clinical applications [201]. Non-viral vectors are less dangerous and may have a more direct path to clinical translation in bone tissue regeneration [202]. CaP biomaterials, especially HA, are promising materials that could serve as non-viral vectors for gene therapy due to their osteogenic properties and high affinity for nucleic acid binding through electrostatic interaction with the  $\text{Ca}^{2+}$  ions at the CaP carrier surface [184,200,205–209]. To be an efficient

DNA and RNA carrier, CaPs particles must be stable in the harsh extracellular environment to protect for the molecular cargo [200]. Moreover, CaP properties like particle size, surface charge and shape are pivotal factors to ensure the absorption of particles containing genetic systems by cells. Olton et al. [209] demonstrated that CaP particle size of about 25 - 50 nm are more effective for non-viral gene delivery. Regarding shape, spherical and porous particles are preferred for gene delivery, drug loading, and cell targeting [210]. Although the use of CaP materials (particularly HA) for gene delivery facilitated bone regeneration, transfection efficiency remains very low compared to viral vectors.

Therefore, the impact of HA modification using synthetic and natural polymers [206,207,211], cell-penetrating peptides [212], biodegradable lipids [213] on endocytosis of HA-based carriers and the subsequent endosomal escape was evaluated. Besides, this strategy has been demonstrated to improve cellular uptake as well as transfection in gene delivery for different applications [205,211,214]. Chitosan polymer has been widely used for the surface modification of HA for gene therapy in bone tissue engineering applications due to its cationic character which leads to improved electrostatic attractions between the amino functional group of chitosan and the negatively charged RNA, thus protecting the RNA during cellular uptake and resulting in an enhanced transfection effectiveness [200,206,215]. HA-CS nanocomposite was developed by Wang et al. [215] by a modified dropping method for DNA gene delivery application, and the developed composite did not show any toxicity against the cells. Lari et al. [206] manufactured successfully nano-size range HA-CS loaded with DNA using emulsion technique.

The use of chitosan-glutamine (CS-Gln) has been also demonstrated to improve transfection and efficiency of CaP materials as a gene delivery system, where the high cationic structure of glutamine is explored to enhance the particle charge of the CaP-CS composite particles. Choi et al. [207] developed a stable HA carrier system with improved intracellular retention by

incorporating glutamine-conjugated chitosan within the apatite matrix, with a significant increase in gene transfection. A newly developed gene-activated matrix comprising polyethylene imine/bone morphogenetic protein 2 plasmid complexes and HA-CS microspheres has been studied to promote bone regeneration [184]. The *in vitro* study proved that the plasmids can be released from the gene-activated matrix in a sustained and controlled manner (for up to 21 days). *In vivo* micro-computed tomography and histological staining data demonstrated a significant improvement in bone regeneration at the defect site, suggesting excellent osteoinductivity of the developed composite.

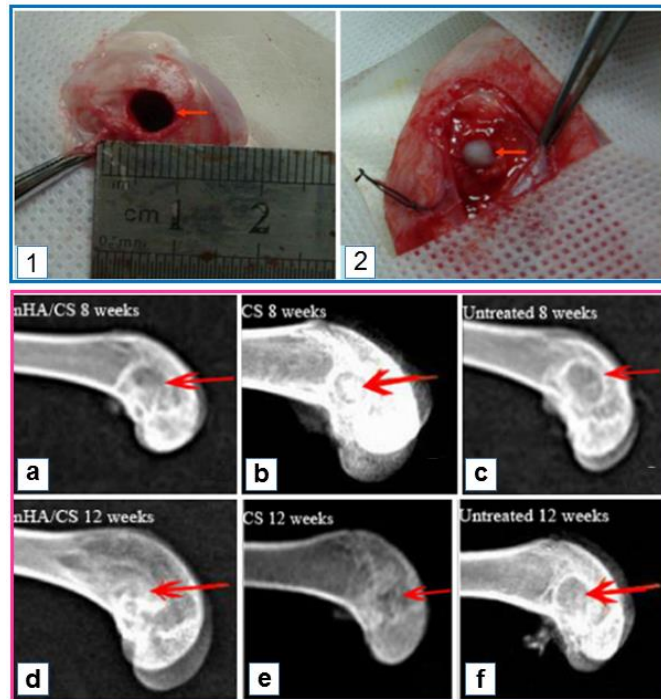
Despite optimistic results were obtained with gene delivery, there is a need to better understand the bone formation process *in vivo*, including the role of DNA/RNA and their interactions in bone physiology. Moreover, the roles and targets of each gene as well as identifying the optimal timeframe for therapeutic administration is required to develop effective and clinically relevant therapies.

## **11. Applications in bone tissue engineering**

Although HA scaffolds produced using different manufacturing techniques have been extensively tested for biomedical applications as bone fractures repair and as drug delivery carriers, recent progress revolutionized this effort resulting in the fabrication of inorganic-organic composites, like hydroxyapatite-chitosan biocomposites with higher porosity, mechanical properties, drug holding capabilities, and biomimetic capacity. The use of HA-CS biocomposites to create scaffolds with high performance has raised drastically due to their biomimetic capacity and improved mechanical and biological properties of the composite compared to separate components. An HA-CS composite scaffold has been made by Zhang et al. [25] through the freeze-drying technique, and its ability to promote bone formation was tested in rabbits models (Fig. 10). The results revealed that most of the defect was filled with the newly formed bone after eight weeks of surgery (Fig. 10a). Besides, more calluses and total

healing of the bone defect were observed in all rabbits after 12 weeks of implantation (Fig. 10d).

The progress on the development of doped-HA-CS is also seen to play an important role in enhancing the biological responses and providing an antibacterial property to the composite. Doping with one or multiple ions has been proved to affect the porosity, crystallinity, mechanical and biological properties of HA and composites based on HA and CS, thus its applicability in the biomedical field [15,160,163,167,168,170]. It has been observed that cationic doping with silver, strontium, copper, zinc, and magnesium could make HA-CS composite antibacterial, anti-osteoporotic and corrosion-resistant without affecting its chemical stability and mechanical integrity. The osteoregenerative ability of bone grafting biomaterials based on Ag doped-HA-CS was evaluated in animals with induced bone defects. Non-critical-size defects in Wistar rats and critical-size defects in the calvarias of guinea pigs were used to examine the biocompatibility and the regenerative capacity of the bones, respectively. Radiographic examination revealed a significant decrease in defects, angiogenesis, and trabecular formation [216].



**Fig. 10.** Surgical procedure: 1) critical size defect was created transversally in the rabbit femoral condyles, 2) defect filled with hydroxyapatite-chitosan (HA-CS) composite. Radiographs of bone repair procedures: (a) in the HA-CS-group, the defect was mostly filled with the newly formed bone after 8 weeks, (d) more calluses were found with a complete bone healing after 12 weeks, (b) in the CS-group, the margin of the defect was not clear with a small new bone formation at 8 weeks, (e) numerous trabecular irregularities were observed at 12 weeks, (c) in the untreated defect, no bone formation was found at 8 weeks, and (f) slight bone formation at 12 weeks. Reprinted from Ref. [25] with permission.

HA and its composites have been extensively utilized as a coating for metallic implant biomaterials [15,103]. Most metallic implants are bioinert and cannot promote cell adhesion and proliferation, which prolongs the healing process of surgical wounds [15]. Such limitations can be resolved using coatings on the implant with biocompatible materials. Wang et al. [217] prepared a BMP-2/HA-CS biocomposite coatings on titanium substrates by one-step micro-arc oxidation combined with dip-coating and encapsulation for bone tissue engineering. The study revealed that the titanium implant material exhibited excellent biological and antibacterial

properties. HA-CS microspheres were prepared through a microfabrication technology and emulsification approach, and loaded the polyethylene imine/bone morphogenetic protein 2 plasmid complexes [184]. *In vivo* results from micro-computed tomography and histological staining revealed that the developed composite, with a nano-network fibrous structure, enhanced bone regeneration in defect areas.

A porous titanium implant with CS-HA coating produced by selective laser sintering technology was *in vivo* investigated [218]. The results showed that the 3D implant promotes the MC3T3-E1 cell's proliferation and differentiation and facilitates osseointegration. The developed composite could be used for dental and orthopedic applications. On the other hand, the use of HA-CS coating to avoid the corrosion of metallic implants has been demonstrated to be efficient. Pang et al. [103] fabricated hydroxyapatite-chitosan composite coatings on 316L stainless steel substrates through the cathodic electrophoretic deposition method. The developed coatings provided anti-corrosion properties for the 316L stainless steel materials.

Several investigations have been performed on the use of CS and HA-CS in the diagnosis and treatment of various tumors owing to their cell membrane penetrability, and pH-dependent therapeutic unloading [219–222]. HA-CS nanofilm was developed for easy and efficient detection of circulating tumor cells in the blood [222]. Improved topographic interactions between the components on cancer cell membranes and the antibody-coated composite led to enhanced circulating tumor cells capture and separation. Circulating tumor cells captured from artificial blood specimens were noted in culture on HA-CS nanofilms components over 14 days using traditional staining technique (hematoxylin-eosin and Wright's stain), suggesting that these composites are capable of isolating and culturing circulating tumor cells for subsequent studies. A novel Au-doped HA-CS film was prepared as an electrochemical immunosensor for the detection of prostate-specific [220]. The developed biosensor was applied to detect prostate-specific antigens in human serum samples, and the obtained data were in good agreement with



enzyme-linked immunosorbent assays. A novel piezoelectric immunosensor using a HA-CS nanocomposite-based biomolecular immobilization technique was investigated for cancer antigen 125 (CA125) detection [223]. The efficiency of HA-CS composite was investigated for the quantification of rare cancer cells in patients with gastrointestinal cancer in clinical use [222]. In fact, the composite allowed the isolation and quantification of cancer cells from cancer patients with a better cell capture yield and could also predict the treatment response.

In bone tissue regeneration, mechanical strength, porosity, surface reactivity and biological properties (biocompatibility, bioactivity) are the main key characteristics that significantly contribute to the design of effective orthopedic implants. Among the developed HA-CS composite scaffolds, minocycline loaded-HA-CS composite with controlled release of minocycline (up to 28 days) developed by Gao et al. [114] appears to be an effective material for periodontal bone defect repair application. Although this material was prepared by a conventional process, *in-situ* precipitation, it presents adequate mechanical strength ( $117.6\pm 31.1 - 242.9\pm 33.6$  MPa) and porosity ( $43.73\pm 2.82$  and  $54.25\pm 1.47$  %). Moreover, it showed a good antibacterial effect against periodontal pathogens (*Staphylococcus aureus* and *Porphyromonas gingivalis*) and more effective bone formation *in vivo*, in rabbit's mandible.

Currently, three-dimensional (3D) printing has become an important tool in the field of tissue engineering, and its further development will lead to completely new clinical possibilities. The use of 3D imprint in the development of bone substitute has been demonstrated to be an attractive alternative to conventional techniques. Combining CaP with polymers through 3D printing technology has the potential to create biomaterials with high performance that may one day compete autologous bone implants. Bioactive 3D-printed scaffolds based on HA and CS with complex architectures were designed by Yousefiasl et al. [126] for personalized craniofacial bone tissue engineering. All the manufactured scaffolds with different HA content (2.5, 5, and 10%) are biocompatible and did not show any toxicity toward rat bone marrow

mesenchymal stem cells. The potential applications of the as-prepared scaffolds are presented in Fig. 11.

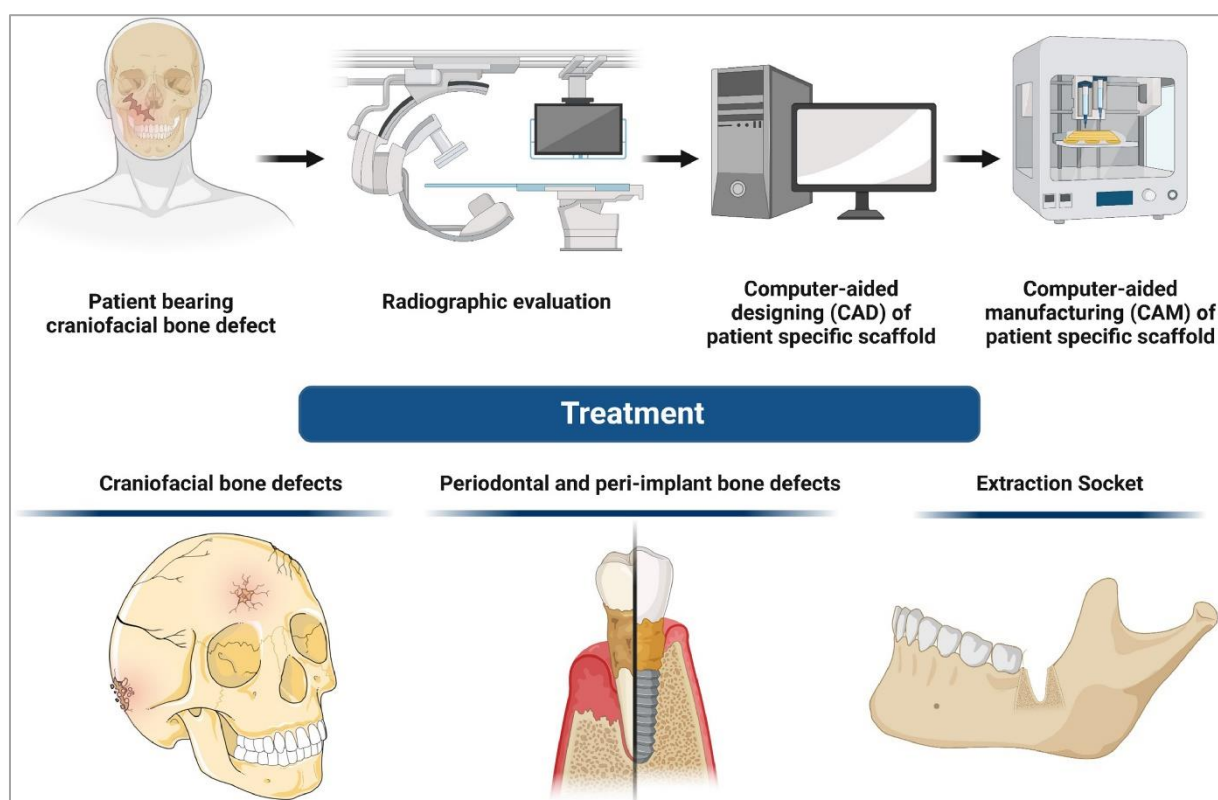


Fig. 11. The potential applications of HA-CS scaffolds manufactured using computer-aided design (CAD) files and computer-aided manufacturing (CAM) by 3D printing [126].

## 12. Conclusions and perspectives

Traditional biomaterials composed of individual compounds suffer from several disadvantages in terms of weak physicochemical and biological characteristics, which tend to limit their use in the biomedical field. Currently, creating organic-inorganic implant materials to efficiently mimic living tissues for bone fracture repair and as drug delivery systems (DDS) is one of the main challenges in chemistry and biomaterial engineering. Biologically inspired hydroxyapatite-chitosan (HA-CS) has been widely investigated in terms of physicochemical, mechanical, and biological properties. Based on the obtained results the greatest similarity of

HA-CS composites to natural bone tissue can be reached by varying the manufacturing methods and the organic/inorganic ratio of the composite. Its biocompatibility, biodegradability, nontoxicity, and bioactivity along with features like mucoadhesion and drug delivery ability have made HA-CS an appreciated composite with numerous applications in the biomedical and pharmaceutical fields. However, the limited mechanical properties and weak antibacterial activity, even in the presence of antibiotics, limits its widespread commercial use. The main factors for unsatisfactory results are as follows: (i) poor integration between components (ii) non-homogeneous structure of the composite or inadequate preparation method and (iii) weak chemical interactions between the carrier and the drug molecules.

This can be circumvented by the development of composite materials based on HA and functionalized chitosan, that could find potential applications in the biomedical field as scaffolds, self-setting formulations, drug carriers, and bone fracture repair. CS modifications like acylation, alkylation, oxidation, thiolation, phosphorylation, sulfation, sulfonation and graft copolymerization with various organic and bioorganic molecules (Fig. 12) could result in composites with higher mechanical properties, increased antibacterial activity, improved hydrophobic interactions, mucoadhesion strength, and the ability for metal ions adsorption, protein and peptide delivery. Recently, special attention has been paid to polymers with phosphorus derivatives in their skeleton, due to their excellent metal chelation, adhesion, and biological properties. In fact, the functionalization of these natural derivatives can further improve the final properties of HA-CS composites (i.e., mechanical strength, chemical reactivity, and biological properties). Investigations and applications of such HA-modified chitosan composite in biomedical, and drug delivery have not yet been addressed.

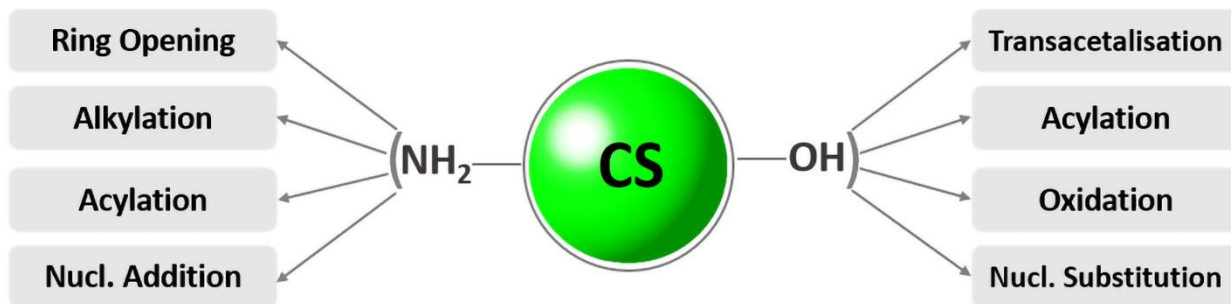
Several attempts have been made to investigate the modified chitosan alone for tissue regeneration with accelerated osteogenesis, faster wound healing, and higher bioavailability of drug delivery with reduced side effects. However, as promising as these studies may seem to

be, the commercialization of the developed functionalized CS has not been successful, which could be due to a lack of controllability over functionalization processes. Thus, the sophistication of the process would be required to control the modification of the polymer with desired substitution degree and high purity. This would certainly address some unmet needs in the biomedical field.

HA is a promising biomaterial with a unique structure, which allows anionic and cationic substitutions by a variety of chemical elements, providing a complex structure with improved properties. Doping of other metals like rare earth (e.g., lanthanum, cerium, praseodymium, neodymium, and samarium) and multi-ionic substitutions might be suitable to provide smart approaches for *in situ* therapeutic applications. New alternative ionic substitutions in the HA-CS matrix, therefore, would be interesting for new biomedical applications.

On the other hand, the development of HA-based biocomposites containing two or several biopolymers in appropriate proportions could open up the possibility of tuning their mechanical and biological properties. Moreover, the development of advanced manufacturing technics like tridimensional bioprinting or additive manufacturing enables the preparation of composite scaffolds with controllable characteristics (internal architecture, porosity, and interconnectivity), highly complex structures, and patient-specific requirements.

One important aspect that deserves consideration and was not explicitly addressed in most previous studies, was the assessment of the interaction (i.e., adsorption and desorption phenomenon) behavior between the drug molecules and the HA-CS carrier surface. This approach appears appealing in view of a better understanding of the mechanisms involved at the drug/carrier interface, consequently controlling the loading and release of the drug molecules.



**Fig. 12.** Functionalized derivatives of chitosan.

### **Acknowledgements**

This research was supported by OCP Foundation through a R&D Initiative «Appel à projets autour des phosphates, APPHOS» under the project ID: MAT-BAR-01/2017. The authors would like to thank OCP Foundation, OCP Innovation, UM6P, CNRST, MESRSFC (Morocco).

**Conflicts of Interest:** The authors declare no conflict of interest.

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**Fig. 1.** Different forms of calcium phosphates biomaterials: (a) Powder for the application in protective coatings, (b) blocks based 3D Scaffold bone substitute materials for filling or reconstructing bone fracture or for filling bone defects, (c) CaP cement used as injectable materials for minimally invasive surgery, (d) coated dental implant and (e) granules used for bone repair or replacement.

**Fig. 2.** Structure of hexagonal hydroxyapatite (space group P63/m) [8].

**Fig. 3.** Schematic representation of hydroxyapatite preparation using : a) Sol-Gel and (b) hydrothermal methods.

**Fig. 4.** Extraction of chitosan from different natural sources.

**Fig. 5.** Example of hydroxyapatite-chitosan composite fabrication through freeze-drying technique, and the surface morphology of the final products using SEM analysis.

**Fig. 6.** Preparation steps of hydroxyapatite-chitosan using *in situ* chemical precipitation.

**Fig. 7.** Tri-dimensional bioprinting techniques commonly used for biomaterials manufacturing: a) Extrusion printing and b) inkjet printing, c) the selective laser sintering and, d) stereolithography [119]. Examples of designed and printed scaffolds: (1) 3D printed scaffolds made of PCL/TCP composite. Reproduced with permission from [129]; (2) dicalcium phosphate anhydrous (DCPA) implant for cranial bone defects repair, and (3) 3D printed scaffolds made of DCPA [8].

**Fig. 8.** Schematic representation of possible chemical interaction between hydroxyapatite and chitosan: (a) Hydrogen bonding (----hydrogen bonds) between the OH group of hydroxyapatite and the —OH and —NH<sub>2</sub> functional groups of chitosan, (b) Coordination bond between Ca(II) of hydroxyapatite and —NH<sub>2</sub> of chitosan.

**Fig. 9.** Surgical procedure: 1) critical size defect was created transversally in the rabbit femoral condyles, 2) defect filled with hydroxyapatite-chitosan (HA-CS) composite. Radiographs of bone repair procedures: (a) in the HA-CS-group, the defect was mostly filled with the newly formed bone after 8 weeks, (d) more calluses were found with a complete bone healing after 12 weeks, (b) in the CS-group, the margin of the defect was not clear with a small new bone formation at 8 weeks, (e) numerous trabecular irregularities were observed at 12 weeks, (c) in the untreated defect, no bone formation was found at 8 weeks, and (f) slight bone formation at 12 weeks. Reprinted from Ref. [25] with permission.

**Fig. 10.** Human colon tumor xenografts in mice performed by injecting HCT 15 cells in the right flank of a nude mouse: (a) tumor-inhibiting effect of tail vein injections of celecoxib, HA-CS, and celecoxib-HA-CS composite on nude mice with HCT 15 xenografted carcinoma cells, (b) tumor volume and (c) image of excised tumors at the sacrifice time (30 days post-treatment). Tumors from the different treatment groups were immunohistochemically analyzed for Ki-67 expression and apoptosis (TUNEL and DAPI). (c) Tumors treatment with celecoxib reduced the number of Ki-67 positive cells and, (d) raised the number of TUNEL-positive cells and (d) nuclear fragmentation. Reprinted from Ref. [177] with permission.

**Fig. 11.** The potential applications of HA-CS scaffolds manufactured using computer-aided design (CAD) files and computer-aided manufacturing (CAM) by 3D printing. Reprinted from Ref. [126] with permission.

**Fig. 12.** Functionalized derivatives of chitosan.