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# A review of chitosan-, alginate-, and gelatin-based biocomposites for bone tissue engineering

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## ABSTRACT

Bone diseases and injuries have a major impact on the quality of life. Classical treatments for bone repair/regeneration/replacement have various disadvantages. Bone tissue engineering (BTE) received a great attention in the last years. Natural polymers are intensively studied in this field due to their properties (biocompatibility, biodegradability, abundance in nature, high processability). Unfortunately, their mechanical properties are poor, which is why synthetic polymers or ceramics are added in order to provide the optimal compressive, elastic or fatigue strength. Moreover, growth factors, vitamins, or antimicrobial substances are also added to enhance the cell behavior (attachment, proliferation, and differentiation). In this review, new scientific results regarding potential applications of chitosan-, alginate-, and gelatin based biocomposites in BTE will be provided, along with their *in vitro* and/or *in vivo* tests.

**Keywords:** *bone tissue engineering (BTE), bone composition, bone functions, biomaterials, natural polymers, chitosan, alginate, gelatin, growth factors, in vitro and in vivo tests***.**

## 1. INTRODUCTION

Tissue engineering received a great attention in the last years due to its innovative approaches regarding the healing of the damaged tissues. Classical treatments do not restore both structural and functional roles of the affected tissue, as in most cases the final structure is just similar to the healthy tissue/organ and the functions are not at their full capacity.

The bone tissue has a high capacity to regenerate itself when small defects appear, but in the case of large bone defects, additional help is required. Several diseases such as osteoporosis, cancer, and osteoarthritis, along with fractures and bone infections, are currently aspects that affect life quality. This explains why bone is the second transplanted tissue, after blood (Roseti et al., 2017).

The gold standard for bone replacement is still represented by autografts, but disadvantages such as the limited sources, resorption, or their high rate of failure are the reason for which novel bone tissue engineering (BTE) approaches are required. Moreover, allografts or xenografts are also used in this filed, but issues such as pathogen transmission or rejection due to immune responses limit their use. Metal implants are also a current approach to ensure, for example, the locomotor function of the bones. The main disadvantage is that metals form an adjacent fibrous tissue when implanted in the body, and the incidence of failures is increased due to this reason. Moreover, metals are non-degradable materials and the regeneration of the bone is stopped after implantation. Furthermore, even if in the first years the implantation is declared as a success, after a long period a new intervention is required because metal implants have a specific life-time (Roseti et al., 2017, Vijayavenkataraman et al., 2018, Turnbull et al., 2018).

Regenerative medicine and tissue engineering received great scientific attention in the last years as they could provide solutions to overcome the limitations of the classical treatments presented above. Regarding BTE, a biomaterial is a "*temporary matrix that promote a specific environment and architecture for bone growth and development"*(Roseti et al., 2017).

Several scaffolds are currently studied due to their great advantage to assure a 3D support for cells proliferation and differentiation. Their characteristics (porosity, degradability, mechanical properties, and surface chemistry) significantly influence the final success of the biomaterial. In an ideal situation, a scaffold used in BTE should have optimized properties to ensure cell growth, nutrients and metabolic waste transport, a degradation rate that match with the regeneration rate of the bone, and mechanical properties similar to those of the surrounding tissue at the affected site. Moreover, the cells should be able to attach, proliferate, and differentiate into and onto the scaffold (Tajbakhsh and Hajiali, 2017, Turnbull et al., 2018).

Unfortunately, it is not possible to check all these criteria, but new scientific outcomes revealed that several biomaterials should be considered for pre-clinical and clinical tests due to their promising *in vitro* and *in vivo* results. Polymeric and ceramic-based biomaterials are studied for their potential in BTE. A new tendency regarding the use of natural substances is observed in the scientific field. In this review, new scientific results regarding chitosan, alginate and gelatin-based biocomposites for BTE will be presented.

## 2. BONE TISSUE ENGINEERING

As mentioned above, the classical treatments for bone tissue injuries/damages have many disadvantages. Therefore, BTE received great attention in the last years. Scientific researchers have studied many biomaterials, along with a variety of synthesis methods, in order to achieve as many as possible of the required properties for bone tissue replacement materials (Turnbull et al., 2018, Tajbakhsh and Hajiali, 2017).

It is known that it is not yet possible to check all the desired properties for a scaffold proposed for BTE applications. Biocompatibility is anyway necessary. The biomaterial should not induce an immune rejection after implantation. Moreover, the biodegradability of the scaffold is also important. In order to assure a support for tissue growth, the scaffold degradation rate should be similar to the regeneration rate of the bone and after degradation, the byproducts should not induce toxic effects inside the body. BTE scaffolds should be bioactive to ensure osteoconduction (assure new bone growth on the scaffold), osteoinduction (induction of osteogenesis), osseointegration, and vascularization processes. Furthermore, the porosity of the scaffold must be interconnected to ensure cell diffusion and migration and the transport of nutrients and metabolic waste. Micro- and macropores (>100 μm) are required to ensure cell-scaffold interactions and neovascularization at the affected site. Natural polymer-based biomaterials should possess all these requirements, along with specific mechanical properties (compressive, elastic, and fatigue strength) (Turnbull et al., 2018, Preethi Soundarya et al., 2018b).

In BTE, proteins, peptides, cells, chemicals, or growth factors are added into the scaffolds to enhance their properties and assure their potential for clinical applications. Mesenchymal stem cells (MSCs) are used in BTE due to their ability to differentiate into several cell types and secrete cytokines (with anti-inflammatory activity) (Preethi Soundarya et al., 2018b).

In order to obtain improved BTE biomaterials, it is important to know the properties of bone, which will be briefly presented in the next chapter.

## 3. BONE PROPERTIES

Bones are part of the skeletal system. Their main functions (Table 1) include the support/protection of the soft tissues and organs, mineral storage (calcium, phosphate), locomotion, and homeostasis. Bone composition (Table 1) is heterogeneous and is represented by inorganic (hydroxyapatite), organic components (type I collagen, lipids and non-collagenous proteins), and water, along with support and remodeling cells (Turnbull et al., 2018, Roseti et al., 2017, Rao et al., 2018, Lowe and Anderson, 2015).

There are two structural types of bone: trabecular (cancellous/spongy bone) and cortical bone, and two types of bone formation: intramembranous (the differentiation of mesenchymal stem cells into bone cells) and endochondral ossification (a cartilage is formed, vascularized, and, due to osteoprogenitor cells, is replaced with bone) (Table 1) (Rao et al., 2018, Wubneh et al., 2018, Ralston, 2017).

**Table 1.** Bone functions, composition, structural, and bone ossification types

| **BONES** | |
|---|---|
| **Functions** | Soft tissue support/protection |
| | Mineral storage |
| | Locomotion |
| | Homeostasis |
| **Composition** | Inorganic components (Hydroxyapatite – 70%) |
| | Organic components (collagen, lipids, proteins) |
| | Water |
| | Support cells (osteoblasts; osteocytes) |
| | Remodeling cells (osteoclasts) |
| **Structural types** | Trabecular bone |
| | Cortical bone |
| **Bone formation types** | Intramembranous ossification |
| | Endochondral ossification |

The trabecular bone has a low density, a high surface area, and presents spaces filled with bone marrow. It has a low elastic modulus, a 30-90% porosity, and contains organic components. Most metabolic activities take place at this level and nutrients and metabolic waste can be transported. The cortical bone has a higher density, but a smaller surface area and surrounds the marrow cavity. It consists in Haversian systems – concentric lamellae of bones. A Haversian system surrounds a canal with blood vessels. The cortical bone has a lower porosity (5-30%) and a higher elastic modulus due to the absence of organic components and an increased mineral content (Ralston, 2017, Wubneh et al., 2018). It is also important to mention that in BTE the mechanical properties of the final product should be considered and should match to those of natural bone (Table 2).

**Table 2.** Mechanical properties of cortical and trabecular bone

| Bone Type | Young's modulus (GPa) | Compressive strength (MPa) | Reference |
|---|---|---|---|
| **Cortical** | 15-20 | 100-200/130-225 | (Balagangadharan et al., 2017)/ (Wubneh et al., 2018) |
| **Trabecular** | 0.1-2 | 2-20/7-10 | (Balagangadharan et al., 2017)/ (Wubneh et al., 2018) |

Bone has the capacity to regenerate itself. When it is destroyed, new bone is formed through the action of various physical and hormonal factors. Bone cells have an important role in bone remodeling. There are four main types: osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts. The osteoprogenitor cells are stem cells which differentiate into osteoblasts or osteocytes. Osteoblasts have an important role in the osteoid synthesis, as the organic part of the osteoid is assured by osteoblasts, and osteocytes, which are inactive osteoblasts that can be found in the mineralized bone. Furthermore, osteoclasts are present on the bone surface and act as bone resorption cells (Lowe and Anderson, 2015).

The bone architecture and functions can be affected by several diseases or fractures. For instance, in osteoporosis a decrease of the cortical and trabecular bone was observed, while the damage of both structural bone types appears in fractures. Moreover, bone cancer can induce severe damage to the trabecular bone which could easily lead to pathological fractures. Furthermore, maldevelopment (e.g.: achondroplasia) or other metabolic bone diseases, beside osteoporosis, such as osteomalacia (adults) or rickets (children) and Paget's disease explain why BTE represents an important area in the biomedical field.

## 4. NATURAL POLYMER-BASED BIOMATERIALS FOR BONE TISSUE ENGINEERING

In BTE, the key to success is represented by a similarity between the biomaterial and the extracellular matrix. Several synthetic materials were developed to assure, for example, a specific porosity or degradation rate. (Lalzawmliana et al., 2018).

Polymeric and ceramic-based biomaterials received great attention in BTE. Natural polymers such as chitosan, alginate, or gelatin are studied for BTE applications because they induce a minimal immunologic response and they have increased biocompatibility properties, which lead to enhanced cell behavior (Lalzawmliana et al., 2018, Rao et al., 2018). Due to their disadvantage, such as low mechanical properties, natural polymers can be mixed with bioceramics or with other synthetic polymers to obtain composite biomaterials with enhanced properties (Wubneh et al., 2018).

In this chapter, new scientific discoveries regarding chitosan, alginate and gelatin-based biomaterials are presented.

### 4.1. Chitosan-based biomaterials

#### 4.1.1. Chitosan properties

Among the biomaterials studied for BTE, chitosan has received great attention due to its biocompatibility, biodegradability, and osteoconductivity properties. Chitosan is a natural polymer, a linear polysaccharide, composed of N-acetyl glucosamine and glucosamine units. This units are linked by β(1-4) bonds. Furthermore, chitosan is obtained through the deacetylation process of chitin, which is abundant in nature. Chitin can be found in bacteria and fungi cell walls, but it is usually extracted from crustacean exoskeleton. Chitosan processability is another advantage for BTE, which is why several forms including films, fibers, or sponges are intensively studied for orthopedic applications. Moreover, the Food and Drug Administration (FDA) approved the use of chitosan in wound dressing (LogithKumar et al., 2016, Balagangadharan et al., 2017, Preethi Soundarya et al., 2018a).

Despite these advantages, low osteoinductivity and low mechanical properties of chitosan biomaterials were signaled by scientific researchers. To address these issues, nanomaterials such as nanohydroxyapatite, nanobioactive glass ceramics, zirconia, silicon dioxide nanoparticles and other polymers were added to chitosan and promising results were revealed regarding the enhancement of the above-mentioned properties (LogithKumar et al., 2016, Balagangadharan et al., 2017).

However, the pH dependent solubility of chitosan and its rapid *in vivo* degradation limit the use of chitosan-based biomaterials. Moreover, the polycationic nature of this polymer significantly affects its hemocompatibility, the antimicrobial activity of chitosan is influenced by the pH of the environment, and, because of its structure (no charged/reactive groups), the bioactivity of chitosan is insignificant (LogithKumar et al., 2016).

#### 4.1.2. New approaches for chitosan-based biomaterials

In the last years, modified chitosan such as quaternized, carboxyalkyl, hydroxyalkyl, phosphorylated or sulfated chitosan had been studied (Table 3) (LogithKumar et al., 2016).

For example, trimethyl chitosan (TMC) has better antimicrobial activity for *E. coli* and *S. aureus* than simple chitosan. Scientific results indicate that for Gram-negative/Gram-positive bacteria, the lipopolysaccharide anionic groups/lipoteichoic acids can create electrostatic interactions with TMC groups at molecular level. In these conditions, the bacteria membrane is destroyed. However, it is important to mention that the antibacterial properties diminished when the pH decreased (LogithKumar et al., 2016).

Similar results regarding the influence of medium pH on antibacterial activity were reported for N-(2-hydroxyl) propyl-3-trimethylammonium chitosan chloride (HTCC). Under alkaline conditions, the antibacterial properties are stronger. For example, HTCC showed higher antibacterial activity against *Streptococcus mutans*, *Prevotella intermedia*,

*Actinobacillusactinomycetemcomitans, Porphyromonas gingivalis*, methicillin-resistant *S. aureus*, and *S. epidermidis* than simple chitosan (LogithKumar et al., 2016).

Biomaterials with antibacterial activity are of great interest because antibiotics have many disadvantages such as: increasing the resistance of bacteria to specific antibiotics, reduce the viability of osteoblasts, or slow down human mesenchymal stem (stromal) cells (hMSCs) proliferation. For example, poly(methyl methacrylate) is a bone cement used in orthopedic applications and, when hydroxypropyl trimethylammonium chloride chitosan (HACC) was added, the differentiation of hMSCs into osteoblasts, the cell attachment, and the apatite formation increased. Moreover, enhanced antibacterial properties were observed (LogithKumar et al., 2016).

Although antibacterial activity is very important in tissue engineering for orthopedic applications, the ability of the biomaterial to induce bone formation remains a priority. For example, hydrogels based on chitosan, HTCC, glycerophosphate, and fibroblast growth factor showed great results regarding bone formation when tested in dogs (LogithKumar et al., 2016).

**Table 3.** Examples of modified derivatives of chitosan (LogithKumar et al., 2016)

| **Quaternized** | **Carboxyalkyl** |
|---|---|
| N, N, N-trimethyl chitosan (TMC) | Carboxymethyl chitosan (CMC) |
| N-(2-hydroxyl) propyl-3-trimethylammonium chitosan chloride (HTCC) | Carboxymethyl chitosan derivatives: |
| hydroxypropyl trimethylammonium chloride chitosan (HACC) | **amino substituted** N-Carboxymethyl Chitosan (N-CMC) |
| N-(2-hydroxyl) propyl-3-triethyl ammonium chitosan chloride (HTEC) | |
| N(2-hydroxyl-phenyl)-N, N-dimethyl chitosan (NHPDCS) | **hydroxyl substituted** O-Carboxymethyl Chitosan (O-CMC) |
| N-(5-chloro-2-hydroxyl-phenyl)-N, N-dimethyl chitosan (NCHPDCS) | |
| N-(2-hydroxyl-5-nitro-phenyl)-N, N-dimethyl chitosan (NHNPDCS) | **amino and hydroxyl substituted** N, O-Carboxymethyl chitosan (N, O-CMC) |
| N-(5bromic-2-hydroxyl-phenyl)-N, N-dimethyl chitosan (NBHPDCS) | Carboxyethyl chitosan |
| N, N, N-(diethyl-p-dimethylaminobenzyl) chitosan (QC3) | Carboxybutyl chitosan |

Carboxymethyl chitosan (CMC) is another example of biomaterial studied for BTE. It has low immunogenicity and improved biocompatibility and biodegradability properties along with better solubility in aqueous solutions. For example, for CMC-hydroxyapatite scaffolds the viability of MC3T3-E1 cells was not affected and the bone formation was higher than in the case of simple hydroxyapatite. CMC deposition on titanium implants showed antibacterial activity against *S. aureus* and *S. epidermidis*. These results are of great value because CMC layers could prevent implant infections which usually lead to implant failure (LogithKumar et al., 2016). Other examples are CMC-poly vinyl alcohol scaffolds, used as hMSCs support, CMC-gelatin-hydroxyapatite injectable gels, used to enhance osteoblasts proliferation, and CMC-gelatin-β-tricalcium phosphate composite, which enhance the mechanical properties. These biomaterials were tested in beagle dogs, in the mandibular region, and showed good results concerning new bone formation (LogithKumar et al., 2016).

CMC derivatives such as N-CMC, O-CMC, or N, O-CMC are also suitable biomaterials for orthopedic applications. Several studies revealed that N-CMC influenced the biomineralization process in a positive way and O-CMC enhanced fibroblast proliferation capacity. For instance, O-CMC-BMP-2 was used to cover the titanium implants surfaces and promising results were achieved regarding their osteointegration. Another example is represented by the N, O-CMC-n-β tricalcium phosphate which was tested in SBF and apatite formation on its surface was observed (LogithKumar et al., 2016).

As mentioned above, besides quaternized and carboxyalkyl chitosan, other modifications of chitosan were presented in scientific research articles. Hydroxypropyl chitosan showed antibacterial activity against *S. aureus* and *E. coli* in a very short period (30 minutes) and hydroxybutyl chitosan was more stable than collagen when it was tested *in vivo*. Phosphorylated chitosan is known for its capacity to bind calcium ions, which lead to enhanced biomineralization properties, and sulfated chitosan presents a high hemocompatibility (LogithKumar et al., 2016).

Other approaches were proposed in scientific literature. For example, chitosan-based nanofibers were studied for their potential in BTE. Several natural and synthetic polymers were added to chitosan to enhance its properties. Polymers such as polycaprolactone, poly(lactic-co-glycolic acid), polycaprolactone-polyvinyl alcohol, and collagen influenced the mechanical strength in a positive manner. Cell adhesion and proliferation were enhanced by polyethylene terephthalate, polycaprolactone core-

shell, polyvinyl alcohol, alginate, or hydroxyapatite addition (Balagangadharan et al., 2017).

Hydroxyapatite-whitlockite composite and chitosan membrane were tested in rabbits to evaluate their effects regarding calvarial defects. The experiment included 12 rabbits, each one with 2 craniotomy defects. For each rabbit one defect was set as a negative control and one was filled with hydroxyapatite-whitlockite powder and covered with a chitosan membrane. The results showed an increase of the expression of osteonectin (this biomarker activity is corelated with the osteogenic activity), early mineralization of the extracellular matrix, and formation of new healthy cortical bone with completely developed osteons compared with the control defects (Luna-Domínguez et al., 2018).

Chitosan-montmorillonite scaffolds were also studied for their potential in BTE. Two types of montmorillonite were used: simple and strontium-modified. The cell viability and proliferation were analyzed. Mitochondrial activity of human primary osteoblasts (hOBs) was evaluated and the results showed that their proliferation on the scaffolds increased in 14 days and their viability was not affected. After 7 days the DNA content was higher for the strontium-montmorillonite scaffold, which is why it was considered that strontium could influence cell proliferation. Moreover, cross-sectional SEM micrographs confirmed that hOBs proliferation was sustained by these scaffolds (Koç Demir et al., 2018).

Hydroxyapatite-chitosan biomaterials proved to be more bioactive than hydroxyapatite. After 28 days in SBF, a layer of apatite was formed on the samples. Other studies confirmed that these composites could be suitable for BTE due to their advantages such as the enhancement of the osteoblast differentiation capacity and their antibacterial properties (Zima, 2018). For instance, MC3T3 cells were cultured on apatite-chitosan and simple chitosan sponges and, after 1 week, the number of cells was higher in the composite scaffolds. Similar results were reported for hydroxyapatite-chitosan scaffolds. For example, pre-osteoblast attachment and pseudopodia expansion were observed on this type of composites. These outcomes, along with other scientific results, indicate that hydroxyapatite could enhance the biocompatibility of the biomaterials studied for orthopedic applications (Jahan et al., 2019).

MG-63 cells were cultured on polycaprolactone-CMC scaffolds with a 12.5% concentration of polycaprolactone. The CMC concertation was varied (5%, 10% and 15%). The MTT assay was used to evaluate the cell viability. The 10% scaffold turned out to be the best support for cell attachment and proliferation, results that are also confirmed by the SEM images. The rapid extension of filopodia was observed after 24 hours. Therefore, polycaprolactone-CMC scaffolds were considered suitable for BTE (Sharifi et al., 2018).

Chitosan-nanohydroxyapatite-resol scaffold is another example of biomaterial tested for BTE. *In vitro* and *in vivo* tests were carried out to evaluate its biological effects. In this regard, aspects such as biocompatibility, biosafety, and degradability were tracked, and the results proved to be promising. MTT assay results indicate that for human osteoblasts MG-63 cells cultured on chitosan-hydroxyapatite-resol scaffolds, the metabolic activity was higher compared to the metabolic activity of the cells cultured on chitosan and chitosan-hydroxyapatite scaffolds. The alkaline phosphatase (ALP) activity of osteoblasts and the protein adsorption were also higher for chitosan-hydroxyapatite-resol scaffolds. *In vivo* experiments were carried out on male Albino rats. The scaffolds were placed into the calvarial defects. The chitosan-hydroxyapatite-resol scaffold proved to be suitable for BTE because it stimulated angiogenesis and soft tissue regeneration. Moreover, compared with a commercial product, this scaffold showed enhanced osteogenic activity and bioactivity (Shakir et al., 2018).

Other studies are also important to mention. For instance, three types of nanocomposites were evaluated for their potential in BTE. Hydroxyapatite, zirconium oxide, and calcium zirconate were added to chitosan solutions in order to obtain three types of scaffolds and to determinate the differences between them. All the composite biomaterials showed higher compressive strength than chitosan scaffolds, especially for zirconium oxide and calcium zirconate. The proliferation of OB-6 pre-osteoblasts was studied for 20 days and the results indicate an increased proliferation for hydroxyapatite and calcium zirconate. These outcomes prove the potential of these scaffolds for BTE (Gaihre and Jayasuriya, 2018).

Poly(L-lactic acid)-chitosan scaffolds were investigated for bone regeneration applications. For comparison, poly(L-lactic acid) and poly(L-lactic acid)-chitosan scaffolds were prepared. To obtain the composite scaffolds, poly(L-lactic acid) samples were immersed in chitosan solutions with 1%, 2%, and 3% concentration. The proliferation of mouse bone marrow stromal cells (mBMSCs) and the ALP activity were higher for poly(L-lactic acid)-chitosan 3%. The *in vivo* experiments were performed in adult male Sprague-Dawley rats. The scaffolds were sterilized and implanted in calvarial defects. After 12 weeks, the results showed that for the poly(L-lactic acid)-chitosan 3%, 90% of the defect was covered with new bone. Therefore, the poly(L-lactic acid)-chitosan 3% scaffold was proposed as a biomaterial with great potential for BTE (Chen et al., 2018).

Chitosan membrane with incorporated chitosan-hydroxyapatite microspheres or simple chitosan microspheres were studied recently for their potential in bone regeneration applications. Human osteosarcoma cells (MG63) were chosen for cytocompatibility tests. On the membranes with chitosan-hydroxyapatite microspheres, the number of cells attached and the filopodia extensions were higher compared with the simple chitosan microsphere membranes. Thus, the results indicate that these types of composite membranes could be suitable for bone defects regeneration (Huang et al., 2018).

Chitosan is a natural polymer which received a great attention for biomedical applications in the last years due to its biocompatibility and antibacterial properties. In BTE studies, chitosan was mixed with other polymers or ceramics to enhance its mechanical properties. The resulting composite biomaterials

showed promising *in vitro* and *in vivo* results, which explain their potential for BTE. The above-mentioned results are summarized in Table 4.

**Table 4**. Scientific results for chitosan-based biomaterials

| Chitosan-based biomaterials | Results | Reference |
|---|---|---|
| TMC | antimicrobial activity for *E. coli* and *S. aureus* | (LogithKumar et al., 2016) |
| HTCC | antibacterial activity against *Streptococcus mutans, Prevotella intermedia, Actinobacillusactinomycetemcomitans, Porphyromonas gingivalis*, methicillin-resistant *S. aureus*, and *S. epidermidis* | |
| poly(methyl methacrylate)-HACC | improved hMSCs attachment and differentiation into osteoblasts, increased apatite formation, antibacterial properties | |
| chitosan-HTCC-glycerophosphate - fibroblast growth factor | new bone formation in dog models | |
| CMC-hydroxyapatite | good viability results and higher bone formation for MC3T3-E1 cells, new bone formation in beagle dog models | |
| CMC-Gelatin-hydroxyapatite | enhanced osteoblasts proliferation, new bone formation in beagle dog models | |
| CMC-Gelatin-β-tricalcium phosphate | enhanced mechanical properties, new bone formation was in beagle dog models | |
| CMC (layer on titanium implants) | antibacterial activity against *S. aureus* and *S. epidermidis* | |
| N-CMC | enhanced biomineralization process | |
| O-CMC | enhanced fibroblast proliferation | |
| O-CMC-BMP2 (layer on titanium implants) | improved osteointegration | |
| O-CMC-n-βTCP | enhanced apatite formation in SBF | |
| chitosan and polycaprolactone/poly(lactic-co-glycolic acid)/polycaprolactone-polyvinyl alcohol/collagen | improved mechanical properties | (Balagangadharan et al., 2017) |
| chitosan and polyethylene terephthalate/polycaprolactone core-shell/polyvinyl alcohol/alginate/hydroxyapatite | enhanced cell adhesion and proliferation | (Balagangadharan et al., 2017) |
| hydroxyapatite-whitlockite and chitosan membranes | high expression of osteonectin, early mineralization of the extracellular matrix, formation of new healthy cortical bone in rabbit models | (Luna-Domínguez et al., 2018) |
| hitosan-strontium modified montomorillonite | improved hOBs proliferation due to strontium content | (Koç Demir et al., 2018) |
| hydroxyapatite-chitosan | enhanced bioactivity, osteoblast differentiation and antibacterial properties, improved MC3T3 cells viability, pre-osteoblast attachment, and pseudopodia expansion; | (Zima, 2018, Jahan et al., 2019) |
| polycaprolactone-CMC | enhanced MG-63 cell attachment and proliferation | (Sharifi et al., 2018) |
| chitosan-hydroxyapatite-resol | improved metabolic activity, ALP activity, and protein adsorption for MG-63 cells and stimulated angiogenesis, enhanced osteogenic activity, and bioactivity in Albino rats | (Shakir et al., 2018) |
| chitosan-zirconium oxide/calcium zirconate/hydroxyapatite | improved compressive strength and proliferation of OB-6 pre-osteoblasts | (Gaihre and Jayasuriya, 2018) |
| poly(L-lactic acid)-chitosan | enhanced proliferation of mBMSCs and improved ALP activity; improved new bone formation in Sprague-Dawley rats | (Chen et al., 2018) |
| Incorporated chitosan-hydroxyapatite microspheres into chitosan membranes | enhanced MG63 attachment | (Huang et al., 2018) |

### 4.2. Alginate-based biomaterials

#### 4.2.1. Alginate properties

Alginate is an anionic natural polymer which received great attention for tissue engineering applications. It is known for its biocompatibility, biodegradability, and hydrophilic properties. In physiological conditions, alginate has the ability to form gels. This property is very important in BTE because the formed porosity allows the cells to populate the network. Moreover, nutrients could easily pass through the biomaterial and ensure a good environment for tissue regeneration (Dalheim et al., 2016, Shaheen et al., 2019).

Alginate is a linear unbranched polysaccharide composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G). As it is composed of M-blocks, G-blocks and MG-blocks, its structure is considered irregular. It is important to mention that the ability of

alginate to form gels is influenced by the length of the above-mentioned blocks. Depending on the source, this ability may vary due to the different block structure and composition (Dalheim et al., 2016, Sukhodub et al., 2018).

An important disadvantage of using alginate as a biomaterial for tissue engineering is represented by the lack of sites for protein adsorption and cell attachment (Dalheim et al., 2016, Shaheen et al., 2019).

#### 4.2.2. New approaches for alginate-based biomaterials

Different polymers or ceramics were mixed with alginate to diminish this limitation and the new composite biomaterials were studied for their potential in BTE applications.

For example, the effect of cellulose nanocrystals in chitosan-alginate-hydroxyapatite scaffolds was recently studied. Three cellulose nanocrystals concentrations were proposed (0.5%, 1%, and 2%). The MG-63 osteoblasts viability was determined through MTT assay. The best results regarding cytotoxicity measurements is represented by the 1% cellulose nanocrystals scaffold. For 3 days the cell viability was almost 100%. After SEM micrographs, it was concluded that this scaffold promote the osteoblast proliferation (Shaheen et al., 2019).

Hydroxyapatite-alginate composites with chlorhexidine, an agent with antibacterial activity, were also studied. Three types of samples were analyzed: dried at 37°C, lyophilized at 55°C, and annealed at 1100°C. The degree of swelling of the samples increased when alginate was added. Even if the annealed samples do not contain any polymer at the end of the annealing process, the alginate content influenced their swelling properties. Furthermore, the shape stability decreased with the increase of alginate content. The drug release was measured in PBS and, after 72 hours, the lyophilized samples released the highest concentration of chlorhexidine. These results indicated that hydroxyapatite-Alginate-chlorhexidine composite could be a potential biomaterial for drug release, especially in dental applications (Sukhodub et al., 2018).

Poly(lactic-co-glycolic acid)-magnesium oxide-alginate core-shell microspheres were studied for their ability to release magnesium ions. The magnesium oxide nanoparticles were coated with 3-(Trimethoxysilyl) propylmethacrylate to reduce the surface energy of the particles and assure their dispersion in the poly(lactic-co-glycolic acid) solution. The oil/water emulsion was used as a method to prepare poly(lactic-co-glycolic acid)-magnesium oxide microsphere and the oil/water/oil emulsion method was used to cover these particles with alginate. Promising results were obtained. MC3T3-E1 pre-osteoblasts were used for *in vitro* studies. Compared to poly(lactic-co-glycolic acid) and poly(lactic-co-glycolic acid)-magnesium oxide microspheres, more cells were found on the core-shell microspheres. MTT assay was used to determinate cell viability which was 30% higher for core-shell microspheres than poly(lactic-co-glycolic acid) microspheres after three days. Sprague-Dawley female rats were used for *in vivo* studies. The defects were filled with each tested biomaterial and the quality and the volume of the new bone was evaluated. After 8 weeks the results indicated that for the core-shell microspheres new healthy bone (75% bone volume) was observed. The percentage of bone volume was under 50% for the other types of microspheres. A very important result was the Young's modulus of the regenerated bone, which was equal to 96% of the value measured for the surrounding bone tissue for the core-shell microspheres and under 71% for the other microspheres. These results indicate that Poly(lactic-co-glycolic acid)-magnesium oxide-alginate core-shell microspheres have a great potential for BTE applications (Lin et al., 2018).

Tetronic-alginate thermo-responsive hydrogels with 17β-estradiol, BMP2 microspheres, and/or plasma rich in growth factors were studied for their potential in osteoporotic bone regeneration. Alginate increased the stability of the scaffolds. Sprague-Dawley female rats were used as animal models for *in vivo* tests. For 25 rats, the osteoporosis was induced by bilateral ovariectomy. For all 50 rats (with or without osteoporosis) cranial defects were created. It is important to notice that the addition of alginate decreased the temperature of gel formation under 37°C. This result was considered an advantage because the surgery time decreased. The analysis showed that the bone mineralization was higher for the rats without osteoporosis than for the ill rats. The study also revealed that the BMP2 improved the response to 17β-estradiol (Segredo-Morales et al., 2018).

α-tricalcium phosphate-sodium alginate and α-tricalcium phosphate-methylcellulose composites were studied to observe the influence of these polymers to α-tricalcium phosphate. It was observed that sodium alginate and methylcellulose have different effects on α-tricalcium phosphate. The hydrolysis of tricalcium phosphate to hydroxyapatite was slowed down by the α-tricalcium phosphate-alginate, but not for the α-tricalcium phosphate-methylcellulose when tested in air. In SBF, the results were different: both materials hydrolyzed to hydroxyapatite. The alginate addition to α-tricalcium phosphate increased the compressive strength to a greater extent than the addition of methylcellulose. The results indicate that these biomaterials have a potential for bone regeneration applications (Czechowska et al., 2018).

Sodium alginate and sulfated alginate were used as bioinks in 3D cell printing for delivery of BMP2. MC3T3-E1 osteoblasts and BMP2 were mixed with the bioinks and the 3D samples were obtained by 3D printing. Four types of samples were analyzed (alginate and alginate/sulfated alginate, with three concentrations of sulfated alginate – 0.5, 10, and 30 mg/mL). The addition of sulfated alginate did not affect the rheologic properties of the bioinks and the activity of BMP2 was enhanced for the alginate/sulfated alginate samples, with higher proliferation and osteogenesis. The sample with 10 mg/mL showed the best results regarding metabolic activity, ALP activity, and calcium deposition. The results revealed that alginate/sulfated alginate bioinks are suitable for 3D cell printing and BMP2 delivery for BTE applications (Park et al., 2018).

Strontium effect in alginate foams was recently studied. Macroporous alginate foams were obtained by adding calcium and

strontium carbonate in alginate and sodium bicarbonate aqueous solution. After 24 hours, the gelled disks were washed, frozen, and lyophilized. Five samples were prepared, with different calcium and strontium content. The porosity of the foams was interconnected (pore size: 100-400 μm) and it was not affected by the calcium/strontium molar ratios. The stability of the foams was enhanced for the samples with higher strontium content. For these scaffolds, the proliferation of the hMSCs was sustained for a longer period because the strontium concentration influenced the osteogenic differentiation of these cells. The authors suggested that these strontium modified alginate foams are suitable for future use in BTE (Catanzano et al., 2018).

The interest for natural materials in biomedical applications increased in the last years. Hence, marine calcium carbonate particles were added in alginate hydrogels in order to evaluate their effect on the scaffold properties and cell behavior (cytocompatibility, cell adhesion, differentiation, and proliferation and extracellular matrix mineralization). The marine calcium carbonate powders were obtained from two sources: mussel and oyster. Three types of alginate were used in this study, with different purities of MM and GG blocks. For all the hydrogels the cell viability was acceptable and not affected by the addition of calcium carbonate particles. It was observed that a higher content of G blocks in the alginate used for the hydrogel synthesis led to a decrease in the cell viability. The addition of calcium carbonate particles could enhance the alginate hydrogels stability. Moreover, when oyster calcium carbonate particles were added into the alginate hydrogels, the ALP activity of the hMSCs was improved (Diaz-Rodriguez et al., 2018) .

Silica-sodium alginate biomaterials were also recently studied for their potential in BTE. 3-glycidoxypropyl trimethoxysilane was used to form inorganic/organic covalent bonds, but a small degree of coupling was achieved due to hydrolysis/condensation and diol formation reactions in water. The alginate:3-glycidoxypropyl trimethoxysilane ratios were varied in order to obtain different solutions and hydrolyzed TEOS was added in the final step to ensure the co-condensation. The final samples had 50wt% $SiO_2$ and 50wt% alginate. The aging process lasted for 3 days and the samples were dried for 3 weeks. After that, a number of samples were immersed in calcium chloride dihydrate. This step provided a second crosslinking. The dissolution studies indicate that for the samples with higher content of 3-glycidoxypropyl trimethoxysilane, the silicon release was observed in a shorter period. According to the authors, the co-condensation process between 3-glycidoxypropyl trimethoxysilane and TEOS was not efficient in these conditions or the epoxide groups of 3-glycidoxypropyl trimethoxysilane were transformed into diols, which could lead to an enhanced hydrophilicity of the silica network and the rapid release of silicon in SBF. Regarding the samples with calcium ions, the results indicate that, when tested in TRIS buffer solution, the mass loss for the samples without 3-glycidoxypropyl trimethoxysilane was high in the first 24 h because alginate was linked with the silica network through weak interactions. For the samples with 3-glycidoxypropyl trimethoxysilane, the dissolution was lower, but the addition of calcium ions in these samples decreased the dissolution time. For the 3-glycidoxypropyl trimethoxysilane samples, the compressive strength and the strain to failure enhanced. These results indicated that these biomaterials have potential for BTE (Vueva et al., 2018).

Alginate-based biomaterials are recently studied for their potential in BTE. Recent studies revealed that alginate-polymer, alginate-ceramic, and also alginate-polymer-ceramic composites can promote cell differentiation and proliferation when tested *in vitro*. Moreover, the possibility to add growth factors into these biomaterials and to ensure their release makes these materials a great option for bone regeneration. Furthermore, several studies revealed promising *in vivo* results, which is why alginate-based biomaterials are proposed in many scientific research articles as BTE biomaterials. The above-mentioned results are summarized in Table 5.

**Table 5.** Scientific results for alginate-based biomaterials

| Alginate-based biomaterials | Results | Reference |
|---|---|---|
| chitosan-alginate-hydroxyapatite-cellulose nanocrystals | cell viability almost 100% for MG-63 osteoblasts 1% cellulose nanocrystals | (Shaheen et al., 2019) |
| hydroxyapatite-alginate-chlorhexidine | increased degree of swelling and decreased shape stability due to alginate content | (Sukhodub et al., 2018) |
| poly(lactic-co-glycolic acid)-magnesium oxide-alginate core-shell microspheres | enhanced MC3T3-E1 pre-osteoblasts viability and new bone formation (75% bone volume) with good Young's modulus of the regenerated bone in Sprague-Dawley female rats | (Lin et al., 2018) |
| α-tricalcium phosphate-alginate | increased compressive strength | (Czechowska et al., 2018) |
| alginate/sulfated alginate bioinks | good rheologic properties, improved release and activity of BMP2 (MC3T3-E1 osteoblasts -enhanced proliferation and osteogenesis), good results regarding metabolic and ALP activity and calcium deposition; | (Park et al., 2018) |
| Calcium/Strontium-alginate | enhanced stability and improved proliferation of the hMSCs for higher content of $Sr^{2+}$ | (Catanzano et al., 2018) |
| Calcium carbonate-alginate | improved hydrogel stability and enhanced cell viability for alginate with intermediate content of G blocks | (Diaz-Rodriguez et al., 2018) |
| silica-alginate and 3-glycidoxypropyl trimethoxysilane | rapid silicon release for high contents of 3-glycidoxypropyl trimethoxysilane and decreased dissolution time due to the addition of calcium ions | (Vueva et al., 2018) |

### 4.3. Gelatin-based biomaterials

#### 4.3.1. Gelatin properties

Gelatin is a natural denatured polymer, a derivative of collagen. It is composed of amino acids (hydroxyproline, proline, or sequences such as RGD – arginine-glycine-aspartic acid). Low costs and immunogenicity, aqueous solution solubility, and the different sources of collagen (fish, cattle bone, pig skin) from which it can be extracted are a few examples of the advantages of this protein (Zare Jalise et al., 2018, Moreira et al., 2018).

Gelatin is used in tissue engineering studies due to its biocompatibility and biodegradability properties. Gelatin is considered a suitable biomaterial to mimic the extracellular matrix due to its functional groups and the possibility to form 3D scaffolds with porous structure. Unfortunately, this polymer has a low stability in physiological conditions, which is why several studies evaluated the influence of other substances added into gelatin-based biomaterials. (Azizian et al., 2018).

#### 4.3.2. New approaches for gelatin-based biomaterials

Gelatin-based scaffolds received special attention in the last years. For instance, polycaprolactone-58S bioactive glass-sodium/alginate-gelatin scaffolds were studied recently. In this study alginate-gelatin microspheres were used as a porogen agent. After their dehydration, the porosity of the scaffold increased. The presence of this microspheres in the pores was confirmed by SEM images. Furthermore, the use of 58S bioactive glass-sodium enhanced the mechanical properties, the hydrophilicity, and the biodegradability of the scaffolds (Mao et al., 2018). As mentioned above, alginate is another natural polymer which is currently studied for BTE applications. Considering this, 3D printed alginate-gelatin scaffolds coated with a layer of nanoapatite were recently studied for their potential as bone regeneration biomaterials. Alginate/gelatin bioinks were prepared and the apatite layer thickness was controlled by the content of phosphate ions. The apatite layer increased the Young's modulus of the scaffolds, improved the protein adsorption on scaffolds, and enhanced the rat bone marrow stromal cells rBMSCs adhesion, proliferation, and osteogenic differentiation (Luo et al., 2018).

Furthermore, chitosan was also used to obtain gelatin-based biomaterials due to its properties.

Gelatin-CMC-hydroxyapatite scaffolds were prepared by two methods: high speed stirring induced foaming followed by freeze-drying (SGC samples) and simple freeze-drying (NGC samples – for control). The gelatin and CMC ratios were variated, and the gelatin-CMC-hydroxyapatite solutions were crosslinked with glutaraldehyde, frozen, and lyophilized. The SGC scaffolds presented large pores. Their enzymatic degradation was faster than the NGC degradation in the presence of both collagenase or lysozyme. In the first 3 days the degradation rate was similar. After this period, the SGC degradation increased from 10% up to 55% in the presence of collagenase. On the SGC scaffolds, the proliferation and differentiation of the human Wharton's jelly MSC microtissue enhanced. The results indicated that the macroporous gelatin-CMC-hydroxyapatite scaffolds have a great potential for BTE (Maji et al., 2018).

Novel biomaterials are conceived when scientists try to overcome the known disadvantages of the previous studied biomaterials. For instance, it is known that osteoporosis is related with calcium and vitamins deficiency. In order to solve the damages caused by this disease, several scaffolds were proposed in the scientific literature for bone repair and regeneration. A new approach was recently studied for this purpose. Vitamin D3 was encapsulated into gelatin and mixed with a layered double hydroxides-hydroxyapatite nanocomposite to evaluate the release of Vitamin D3 and the cell cytotoxicity of this biomaterial. Crosslinking was assured by glutaraldehyde addition. A similarity between the porosity of the scaffolds and the bone specific porosity was observed. In the case of Vitamin D3 containing scaffold, the small dimensions of the pores and the thickness of the walls explained its high density and Young's modulus when compared with the Vitamin D3-free scaffolds (with increased porosity). The addition of hydroxyapatite also influenced scaffold properties. For instance, the mechanical strength decreased, and the porosity along with the density increased. For the *in vitro* tests, the G-292 cell line was used. The Vitamin D3 and hydroxyapatite significantly influenced the cells behavior with enhanced cell viability and proliferation. Results were promising, and the authors suggested that the scaffolds are suitable for BTE applications (Fayyazbakhsh et al., 2017).

The influence of hydroxyapatite in gelatin-based biomaterials was intensively studied. For example, hydroxyapatite-gelatin composites were recently studied to evaluate their effect on mBMSCs osteogenic differentiation. A hydroxyapatite nanowires suspension was added in a gelatin solution. The hydroxyapatite:gelatin ratios were varied, and the casting method was used to form the films. The crosslinking was assured by soaking the films into an EDC/NHS solution. It was observed that with the decrease of hydroxyapatite content, the water absorption increased, and with the increase of gelatin content, the water vapor transmission rate decreased. The mechanical properties were also studied in order to evaluate this biomaterials potential for tissue engineering. The Young's modulus decreased with the addition of higher contents of gelatin, but the elongation to break increased. *In vitro* studies revealed that the addition of hydroxyapatite into gelatin involves an increased cellular mineralization and ALP activity. For the samples with a higher content of hydroxyapatite, the calcium concentration and the expression of specific proteins related with the osteogenic differentiation were significantly higher compared to the samples with a higher content of gelatin, but a lower content of hydroxyapatite. The authors suggested that for bone biomedical applications, the composites with a higher content of hydroxyapatite are more suitable (Lian et al., 2018).

Other results regarding the influence of hydroxyapatite, β-tricalcium phosphate and 58s bioactive glass were reported in the literature. The freeze-drying method was used to synthesize gelatin-chitosan based scaffolds. The content of hydroxyapatite, β-

tricalcium phosphate, and 58s bioactive glass was 30wt%. The lowest compressive strength was measured for gelatin-chitosan-bioactive glass and the highest for gelatin-chitosan-hydroxyapatite. Despite that, gelatin-chitosan-bioactive glass scaffold proved to be the best substrate for hMSCs attachment, proliferation, and differentiation when tested *in vitro*. *In vivo* experiments were carried out on adult New Zealand white rabbits. The defects created in the distal femur of each rabbit were filled with the tested scaffolds. The results revealed that gelatin-chitosan-bioactive glass scaffold was the best biomaterial for bone regeneration compared with the others (Dasgupta et al., 2019). Incorporated bioactive glass into gelatin hydrogels were also recently studied. For bioactive glass-gelatin methacryloyl composites synthesis various amounts of bioactive glass were dispersed in gelatin methacryloyl solutions. Two sets of hydrogels were studied: irradiated with UV light after synthesis and after incubation at 4°C. The physical crosslinking was ensured by the gelation step and the chemical crosslinking by the UV step. All the samples presented interconnected porosity and smaller pores were observed for the incubated and UV irradiated hydrogels due to the physical crosslinking. However, when the bioactive glass content increased, the pore size also increased for the incubated and UV irradiated hydrogels. Bioactive glass proved to enhance the stability and bioactivity of the hydrogels and ensure rBMSCs attachment, proliferation, and differentiation (Zheng et al., 2018).

Gelatin methacryloyl-chitosan hydrogels were also recently studied to evaluate the influence of photocrosslinking and basification regarding the enhancement of the mechanical properties. The compressive and tensile moduli along with the tensile stress and strain of the hydrogels with interpenetrating network and semi- interpenetrating network structure were evaluated. Compared with the gelatin methacryloyl hydrogels, the results indicated that the stiffness of the semi-interpenetrating network gelatin methacryloyl-chitosan hydrogels was higher. When photocrosslinking is followed by basification, the chitosan aggregation appeared, and the interpenetrating structure was achieved. With the increase of chitosan concentration, the Young's and compressive modulus increased. Hence, the authors indicate that interpenetrating network gelatin methacryloyl-chitosan hydrogels are as biocompatible as pure gelatin methacryloyl, but have enhanced mechanical properties, which is why they are a promising biomaterial in BTE (Suo et al., 2018).

As mentioned above, the addition of bioactive into gelatin-based biomaterials seems to enhance cell behavior. For instance, chitosan-gelatin-bioactive glass hydrogels were studied to enhance their injectability and to evaluate their potential in BTE applications. The gelatin:chitosan ratios and the amount of bioactive glass were varied. The results revealed that these hydrogels are suitable for drug and cell encapsulation. At physiological temperature and pH, these composites are able to form a gel. Human osteosarcoma cell line (SAOS) viability was observed and the final results indicated that all the tested formulas are biocompatible, with no cytotoxic effects. However, it was observed that the gelatin and bioactive glass contents enhanced cells behavior. Additionally, the injectability of these hydrogels was evaluated and the authors suggested that it was suitable for biomedical applications (Moreira et al., 2018).

Other chitosan-gelatin based biomaterials have recently been studied. Porous chitosan-gelatin scaffolds with chitosan nanoparticles containing basic fibroblast growth factor and bovine serum albumin were recently synthetized. It was observed that the porosity of the scaffolds increased when the chitosan nanoparticles were present. This aspect and the hydrophilicity of the chitosan nanoparticles explained the higher swelling ratio and water uptake capacity of the final scaffolds. Regarding the *in vitro* tests, the samples containing bovine serum albumin and basic fibroblast growth factor assured a higher cell viability and an enhanced proliferation of normal human dermal fibroblasts. The authors suggested that these scaffolds are suitable for tissue engineering applications, especially when angiogenesis is needed, and that the stability of these biomaterials could be enhanced by adding crosslinkers (Azizian et al., 2018). The angiogenesis is also important in bone tissue regeneration. For this reason, these growth factors delivery scaffolds should be further studied for BTE applications using suitable growth factors for bone regeneration.

As mentioned above, strontium effect was studied in chitosan- or alginate-based biomaterials. Similar studies were proposed for gelatin-based composites. Furthermore, due to the positive effects of bioactive glass regarding cell behavior, strontium-gelatin-bioactive glass composites were recently studied. The freeze-drying method was used to synthesize the scaffolds. *In vitro* tests were performed on rBMSCs isolated from Wister rats. The samples containing strontium had a higher stiffness, ALP activity, and an enhanced angiogenesis. These results indicated that strontium-gelatin-bioactive glass scaffolds have potential for BTE applications (Zare Jalise et al., 2018).

Gelatin is a biopolymer intensively studied for its properties. Due to its advantages, gelatin-based biomaterials are more and more studied in order to obtain the best properties for BTE. The above-mentioned results are summarized in Table 6. Other polymers and even ceramic biomaterials are added into gelatin to assure the mechanical properties and the stability of gelatin-based biomaterials. Promising results were provided by scientific researchers regarding *in vitro* and *in vivo* tests, but pre-clinical and clinical tests are required to ensure the entry of these biomaterials in the biomedical field.

**Table 6.** Scientific results for gelatin-based biomaterials

| Gelatin-based biomaterials | Results | Reference |
|---|---|---|
| polycaprolactone-58S bioactive glass-sodium/alginate-gelatin | enhanced mechanical properties, hydrophilicity, and biodegradability | (Mao et al., 2018) |
| alginate/gelatin bioinks with an apatite | increased Young's modulus, protein adsorption, and enhanced | (Luo et al., 2018) |

| | | |
|---|---|---|
| layer | rBMSCs adhesion, proliferation, and osteogenic differentiation | |
| gelatin-CMC-hydroxyapatite | enhanced proliferation and differentiation of human Wharton's jelly MSC microtissue/slower degradation | (Maji et al., 2018) |
| Vitamin D3 encapsulated in gelatin/layered double hydroxides-hydroxyapatite | high density and Young's modulus and: enhanced cell viability and proliferation for G-292 cells | (Fayyazbakhsh et al., 2017). |
| hydroxyapatite-gelatin | decrease of hydroxyapatite content – increased water absorption; increase of gelatin content – decreased water vapor transmission rate, Young's modulus, and increased elongation; high contents of hydroxyapatite – increased cellular mineralization, ALP activity, calcium concentration, and expression of specific proteins related with the osteogenic differentiation (mBMSCs) | (Lian et al., 2018). |
| gelatin-chitosan-bioactive glass | low compressive strength, enhanced hMSCs attachment, proliferation, and differentiation, and good *in vivo* results | (Dasgupta et al., 2019) |
| bioactive glass-gelatin methacryloyl-chitosan | higher stiffness for semi-interpenetrating hydrogel networks; increase of CS concentration – increase of Young's and compressive modulus; | (Suo et al., 2018) |
| chitosan-gelatin-bioactive glass | suitable for drug and cell encapsulation, good human osteosarcoma cell line viability | (Moreira et al., 2018) |
| chitosan-gelatin-chitosan nanoparticles and bovine serum albumin and basic fibroblast growth factor | high swelling ratio and water uptake capacity, high cell viability and enhanced proliferation of normal human dermal fibroblasts, enhanced angiogenesis; | (Azizian et al., 2018) |
| Strontium-gelatin-bioactive glass | high stiffness, ALP activity, and an enhanced angiogenesis | (Zare Jalise et al., 2018) |

## 5. CONCLUSIONS AND FUTURE PERSPECTIVES

The classical treatments for bone tissue injuries have many disadvantages such as limited sources or resorption (autografts), immune rejection (allografts or xenografts), and fibrous tissue encapsulation (metallic implants). BTE biomaterials are intensively studied in order to replace these conventional treatments.

The porosity and biodegradability, along with the mechanical properties and surface chemistry, are important requirements for BTE biomaterials. Several composites where proposed in the scientific research papers as potential substituents for bone tissue. Natural polymer-based biomaterials received a great attention in the last years due to their biocompatibility and biodegradability properties. Moreover, their high processability and abundance in nature represent significant advantages.

Chitosan-, alginate-, and gelatin-based composites revealed promising results for BTE applications. The addition of other synthetic or natural polymers, ceramics, antibacterial substances, or growth factors enhanced the cell behavior (attachment, proliferation, and differentiation), which led to promising *in vitro* and *in vivo* results. Moreover, they improved the mechanical properties and the degradation rate, which are fundamental for BTE applications.

To assure the success of BTE composites, *in vivo* tests should be carried out more frequently. Moreover, more animal models should be taken into consideration for *in vivo* tests, to assure relevant results for future research. Furthermore, pre-clinical and clinical tests are required in order to facilitate the entrance of BTE biomaterials in the biomedical area. If promising BTE biomaterials would be approved as treatment methods, the evolution of biomedical field would be enormous.

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## 7. CONFLICTS OF INTEREST

The author declares no conflict of interest.

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