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Review

Application of Mineralized Chitosan Scaffolds in Bone Tissue Engineering

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Abstract: Chitosan (CS) is a natural cationic polysaccharide obtained via the N-deacetylation of chitin. It has various outstanding biological properties such as nontoxicity, biodegradability, biocompatibility, and antimicrobial properties. Minerals can be deposited on the CS template using different methods to construct composites with structures and functions similar to those of natural bone tissue. These ideal scaffolds can produce bone via osteogenesis, osteoinduction, and osteoconduction, with good biocompatibility and mechanical properties, and are thus considered promising novel biomaterials for repairing hard tissue defects. In the last decade, the field of mineralized CS scaffolds has provided novel fundamental knowledge and techniques to better understand the aforementioned fascinating phenomenon. This study mainly focused on the basic structures and properties of mineralized CS scaffolds to understand the current research progress and explore further development. Further, it summarizes the types, preparation methods, components, properties, and applications of mineralized CS scaffolds in bone tissue engineering during the last 5 years. The defects and shortcomings of the scaffolds are discussed, and possible improvement measures are put forward. We aimed to provide complete research progress on mineralized CS scaffolds in bone tissue engineering for researchers and clinicians, and also ideas for the next generation of mineralized CS scaffolds.

Keywords: chitosan; bone tissue engineering; regeneration; scaffold; mineralization

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1. Introduction

Bone defects caused by aging, trauma, tumor resection, and osteonecrosis remain a major concern in clinical research, leading to disability, health loss, and impaired quality of life, and pose a serious economic burden [1]. At present, the main methods used for treating large-sized bone defects are autologous and allogeneic bone grafts [2]. Autologous bone grafts are considered the gold standard for bone tissue repair. However, some unavoidable defects are encountered, such as infection, inflammation, limited donor bone transplantation, and donor-site neurovascular injury [3]. Allogeneic bone grafts have been developed gradually in recent years; unfortunately, some problems occur even with the use of allogeneic bone grafts, such as immune rejection, blood disease transmission, and poor osseointegration [4]. Bone tissue engineering (BTE) has attracted immense attention because of its great potential to treat critical-sized bone defects and related diseases [5]. BTE refers to the implantation of artificial scaffolds into the site of bone defects, with the synergistic effect of biomaterial, cell, and factor, inducing new functional bone regeneration and repairing the bone tissue defects while the scaffold is degraded step by step [6,7]. Researchers have tried

to improve bone regeneration with different biomaterials. This strategy has the advantages of high controllability, low risk of infection, excellent biocompatibility, and no obvious complications [8–10]. Designing an ideal artificial scaffold is the core of BTE.

Chitosan (CS) scaffolds have been widely used in the field of BTE [11]. CS, a natural cationic polysaccharide, is obtained by the N-deacetylation of chitin and composed of D-glucosamine and N-acetyl-D-glucosamine (Figure 1). It widely exists on earth and is easy and cheap to prepare as scaffolds. The chemical structure of CS is similar to the extracellular matrix (ECM) [12,13]. CS has various outstanding biological properties such as nontoxicity, biodegradability, biocompatibility, and antimicrobial properties [12], while its scaffold is weak in an aqueous environment and limited by the lack of osteoinduction and osteoconduction [14]. However, natural bone is an organic–inorganic complex, comprising of oriented hydroxyapatite and regular arrangement of type I collagen (Col), which have different effects on cells [15]. The ECM in bone tissue also plays an important role in regulating the biological behavior of all kinds of bone cells, in which bioactive factors strengthen bone formation and play a significant role in regulating ossification. Taken together, biomimetic artificial scaffolds should not only be porous but also include similar components and other microstructures of bone tissue, as well as the bioactive factors. The minerals are deposited on the CS template using different mineralization technologies to construct composites with structures and functions similar to those of natural bone tissue. Previous studies have shown that chitosan has good biocompatibility combined with inorganic minerals [16,17]. Due to the unique properties of chitosan, such as nontoxicity, biocompatibility, and biodegradability, chitosan prepared by different assembly methods with specific properties has been widely applied in different tissue regeneration studies [18]. In addition, modified chitosan derivatives with novel properties, functions, and applications have been developed and widely applied in drug delivery and gene therapy recently [19]. This ideal bone-mimetic structural scaffold can produce bone via osteogenesis, osteoinduction, and osteoconduction, with good biocompatibility and mechanical properties [20].

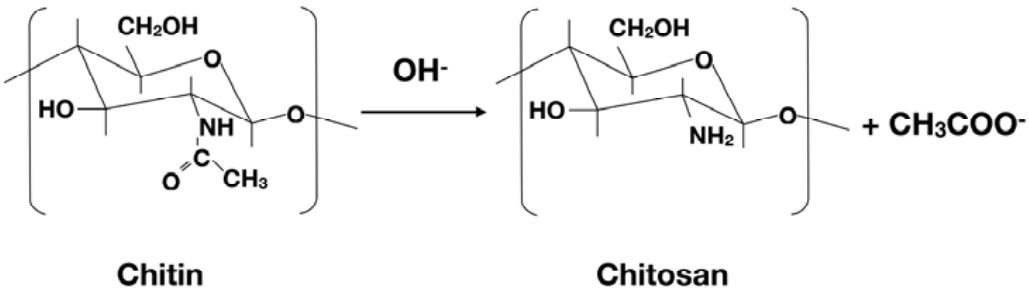

Figure 1. Structures for chitin and chitosan.

Mineralized CS scaffolds are feasible and effective in bone repair, and hence serve as promising scaffolds for bone tissue regeneration. However, the scaffolds are still in the primary stages of research. This study focused on the construction and properties of mineralized CS scaffolds, and examined the types, preparation methods, components, properties, and applications of mineralized CS scaffolds in BTE over the last 5 years to understand the current research progress and explore the means and ways for its further development. The defects and shortcomings of the scaffolds are discussed, and possible improvement measures are put forward. The mineralized CS scaffolds should aim to promote bone repair, rather than simulating the structure of natural bone tissues indiscriminately. Natural bone tissues are extremely complex, and the technologies for artificially synthesizing bone-like scaffolds are limited. Mineralized chitosan scaffolds should be based on the existing technologies and conditions, aiming at promoting bone formation rather than pursuing similar natural bone structures. This study aimed to provide complete research progress to date on mineralized CS scaffolds in BTE for researchers and clinicians and also ideas for the next generation of mineralized CS scaffolds.

2. Structure and Properties of Mineralized CS Scaffolds

Composed of a CS organic template and inorganic minerals, the mineralized CS scaffolds mimic natural bone tissue to obtain excellent biological performance and mechanical properties. Hence, we reviewed the construction and properties of the scaffolds from two aspects: the organic template and the inorganic component.

2.1. Organic Template—CS

CS is a natural cationic polysaccharide obtained via the N-deacetylation of chitin, which is the most widely occurring biopolymer in nature after cellulose, and can be found in crustaceans, fungi, tachypleus tridentatus, insects, and so forth [21]. The degree of acetylation of CS can be characterized by the percentage of acetylation (DA%) [22–24]. When the DA% is less than 50 mol%, chitin is converted to CS. The solubility of CS is improved due to the presence of a large number of free amine groups [24].

CS is an ideal bone repair material because of its rich source, hydrophilicity, nontoxicity, biocompatibility, biodegradability, and antibacterial activity [25,26]. It is insoluble in general solvents but dissolves due to amino protonation in an acidic medium when pH < 5 [24]. Different assembly methods can be used to prepare CS matrices with different scale structures, such as one-dimensional microspheres, two-dimensional fibers, membranes, and three-dimensional scaffolds (Figure 2). The significant properties of CS facilitate its wide use in BTE [27].

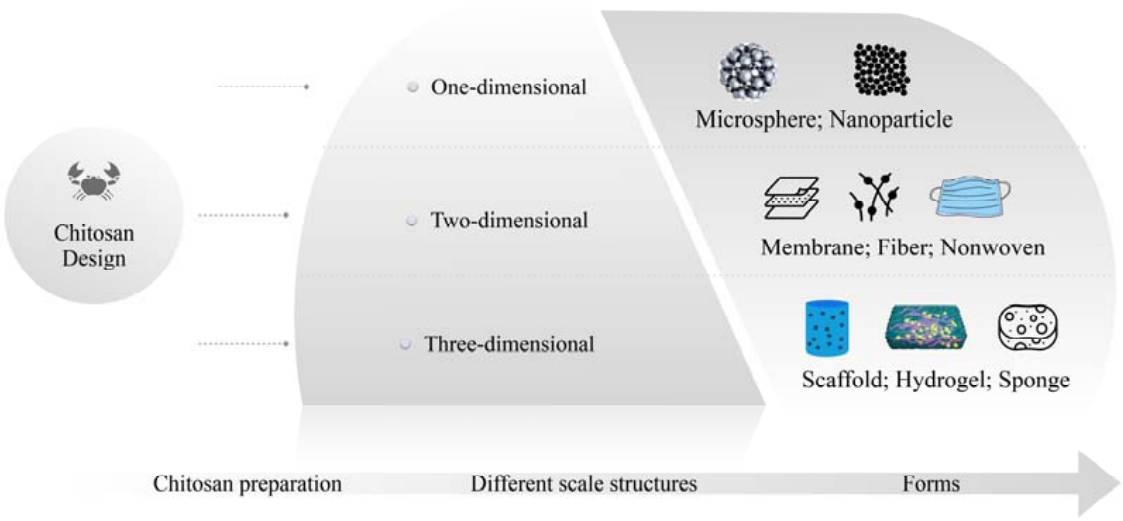

Figure 2. Schematic representation of chitosan formulation into different forms.

2.1.1. Biodegradability

When CS-based materials are implanted, the β-1,4-glucosidic bonds are broken and decomposed into low-molecular-weight CS by enzymes, such as lysozyme and hydrolase, and finally degraded into N-acetyl glucosamine and glucosamine [14]. These two monosaccharides are harmless to the human body and can be completely absorbed. After a series of chemical reactions, some reactants are excreted from the respiratory tract in the form of CO_2, while others are used by the human body in the form of glycoproteins [28,29]. The degradation rate can be controlled by changing the crystallization of CS, which can be modulated by the degree of deacetylation [30].

2.1.2. Drug Delivery

One of the advantages of chitosan derivatives is their ability to store, deliver, and release the drugs or chemicals required in tissue regeneration. In addition, they also confer a sustained release effect and improve drug absorption. Chitosan can be prepared into a variety of forms such as microspheres, nanoparticles, hydrogels, etc., for ophthalmic, oral,

nasal, and colon delivery. Moreover, with the delivery of anticancer agents, chitosan has been applied in cancer treatment by inhibiting the growth of cancer cells. Chitosan is also a promising biomaterial for gene delivery and vaccine delivery [31,32]. CS contains a large number of free amino groups and positive charges. Hence, it can wrap drugs and a variety of bioactive factors by means of chemical cross-linking and electrostatic adsorption and form a semi-permeable membrane on the surface. This characteristic enables the use of CS as a carrier to prolong the release duration of drugs and growth factors.

2.1.3. Antibacterial Activity

The electrostatic interaction between the positive charge in CS and the negative charge in the cell membrane of bacteria leads to the uneven distribution of the negative charge in the cell membrane, which affects cellular function and disrupts the normal metabolism of bacteria [33].

2.2. Inorganic Component—Minerals

The inorganic minerals in CS-based mineralized scaffolds are usually calcium phosphate (CaP), including hydroxyapatite (HA) [34], β-tricalcium phosphate (β-TCP) [35], their biphasic calcium phosphate (BCP) composites [36], amorphous calcium phosphate [37,38], octacalcium phosphate [39], anhydrous dicalcium phosphate [40], and so forth. Additionally, bioactive glass (BG) is a common inorganic component [41].

2.2.1. Hydroxyapatite

Hydroxyapatite [HA; Ca_{10} $(PO_4)_6$ $(OH)_2$] is the major inorganic compound in human hard tissue. It is abundant and inexpensive and has good biocompatibility. Hence, hydroxyapatite was the most common inorganic mineral used in BTE in previous years [42]. HA is a non-toxic, non-anti-prototype bioactive ceramic material, which has been shown to have good bioactivity, be osteoconductive, and have hard histocompatibility [43]. In addition, it can be integrated into bone without provoking an immune reaction because of its grain size and decomposition of the HA powder [44]. Pure HA is not suitable for clinical application due to its brittleness, poor strength, and slow degradation.

2.2.2. β-Tricalcium Phosphate

TCP has three polymorphs: β-TCP, α-TCP, and α′-TCP, which are distinguished by the crystallization phases and sintering temperature. β-TCP is stable below ~1125 °C and is preferred for BTE because of its outstanding mechanical performance and suitable resorption rate [45]. Its solubility is close to that of bone mineral, and it is not soluble under physiological conditions, but it is resorbed by osteoclasts. Meanwhile, β-TCP has shown high osteoinductive potential in addition to its osteoconductive ability [46–48]. In brief, β-TCP is one of the most promising bone graft substitutes.

2.2.3. Biphasic CaP (BCP)

HA and β-TCP make up BCP by different proportions [49]. The major physicochemical properties of BCP depend on the different proportions of the two phases, and are usually similar to the single phase [50]. The main purpose of BCP is to avoid the disadvantages of single phase, and highlight the advantages to improve the bioactivity, osteoconductivity, and osteoinductivity of the biomaterials, achieving the formation of bone tissue [51]. By manipulating the composition ratio of HA and β-TCP, BCP can optimize the biodegradation rate and enhance the bone repair process, because faster or delayed degradation of biomaterials is both not conducive to bone formation [52].

2.2.4. Bioactive Glass

Originally, Hench et al. successfully developed Bioglass 45S5 called BG, which is composed of 45% SiO_2, 24.5% Na_2O, 24.5% CaO, and 6% P_2O_5 [53]. There are two categories of BG nowadays: Class A BG has both osteoconduction and osteoproduction which leads

to rapid bonding to bone and soft connective tissue; Class B BG bonds slowly only to the bone and only osteoconduction occurs on these materials [54,55]. The bioactivity and osteoinductivity of a class A bioactive material is usually greater than that of class B materials [56]. Once BG encounters the body fluid, the hydrolysis of the silica groups leads to the formation of silanol groups (Si–O–H). Si $(OH)_4$ functions as a matrix for HA to precipitate on the surface [57]. BG has excellent biological activity, biocompatibility, osteogenicity, and angiogenic potential [58,59].

3. Preparation Techniques of Mineralized CS Scaffolds

Generally, the preparation of mineralized CS scaffolds involves the preparation of CS organic templates and the deposition of minerals on the organic templates.

The common techniques for preparing CS-based scaffolds are freeze-drying, electrospinning, gelation by cross-linking, and layer-by-layer (LBL) self-assembly [60] (Figure 3). Freeze-drying is one of the most commonly used technologies for preparing CS-based scaffolds. It involves rapidly freezing the samples to be dried and sublimating them under a high vacuum to remove frozen water [61]. The freeze-drying method can be used to prepare layered or columnar pore structures. The radial pore structures can also be obtained by changing the freezing method and conditions, which have been proven to be beneficial to osteogenesis. Electrospinning is a process of using the electric field to control the deposition of polymer fibers on the target substrate [62]. Aidun et al. fabricated a ternary polycaprolactone/CS/Col scaffold with different ratios of graphene oxide using electrospinning [63]. Gelation by physical or chemical cross-linking is a common way to prepare CS hydrogels [64]. Ionotropic gelation involves physical cross-linking and is one of the most widely used techniques for preparing CS nanoparticles. Ahmed et al. used sodium tripolyphosphate (TPP) as a physical cross-linking agent to prepare CS hydrogels [65]. LBL self-assembly involves the self-assembly of polyanions and polycations by using electrostatic, hydrogen bonding, hydrophobic, and van der Waals interactions [66]. CS can react with other materials via electrostatic force, hydrogen bonds, Schiff base linkage, and other interactions, which theoretically supports the practicability of preparing CS-based composites by LBL manipulation [66]. Mu et al. prepared a multilayer film on a titanium rod using a spin-coater-assisted LBL approach, which was constructed using cationic CS and anionic gelatin. The assembled film could continuously release drugs for almost 2 weeks and promoted bone healing [67]. 3D printing, or additive manufacturing (AM), is a unique layer-by-layer assembly technology which involves the fabrication of the same or different materials via an automated process [68]. Mineralized CS 3D scaffolds exhibit an excellent effect in BTE. 3D printing technology allows the rapid and feasible production of the CS scaffolds. Chitosan/collagen/nano-hydroxyapatite scaffolds prepared with the 3D printing process confirmed suitable structural properties. Loading of crocin into the scaffolds showed lower toxicity [69]. Combining an extrusion printing technique with an impregnation method, researchers created composite scaffolds from chitosan/HA/alginate. The results illustrated that 3D printed scaffolds enhanced the cell viability and attachment [70]. Wei et al. discussed forming quality and mechanical properties of HA/carboxymethyl chitosan (CMCS) composite ceramic scaffolds. Incorporating CMCS into HA enhanced the toughness of the scaffolds, while a 5 wt% content of CMCS led to the poor forming quality [71].

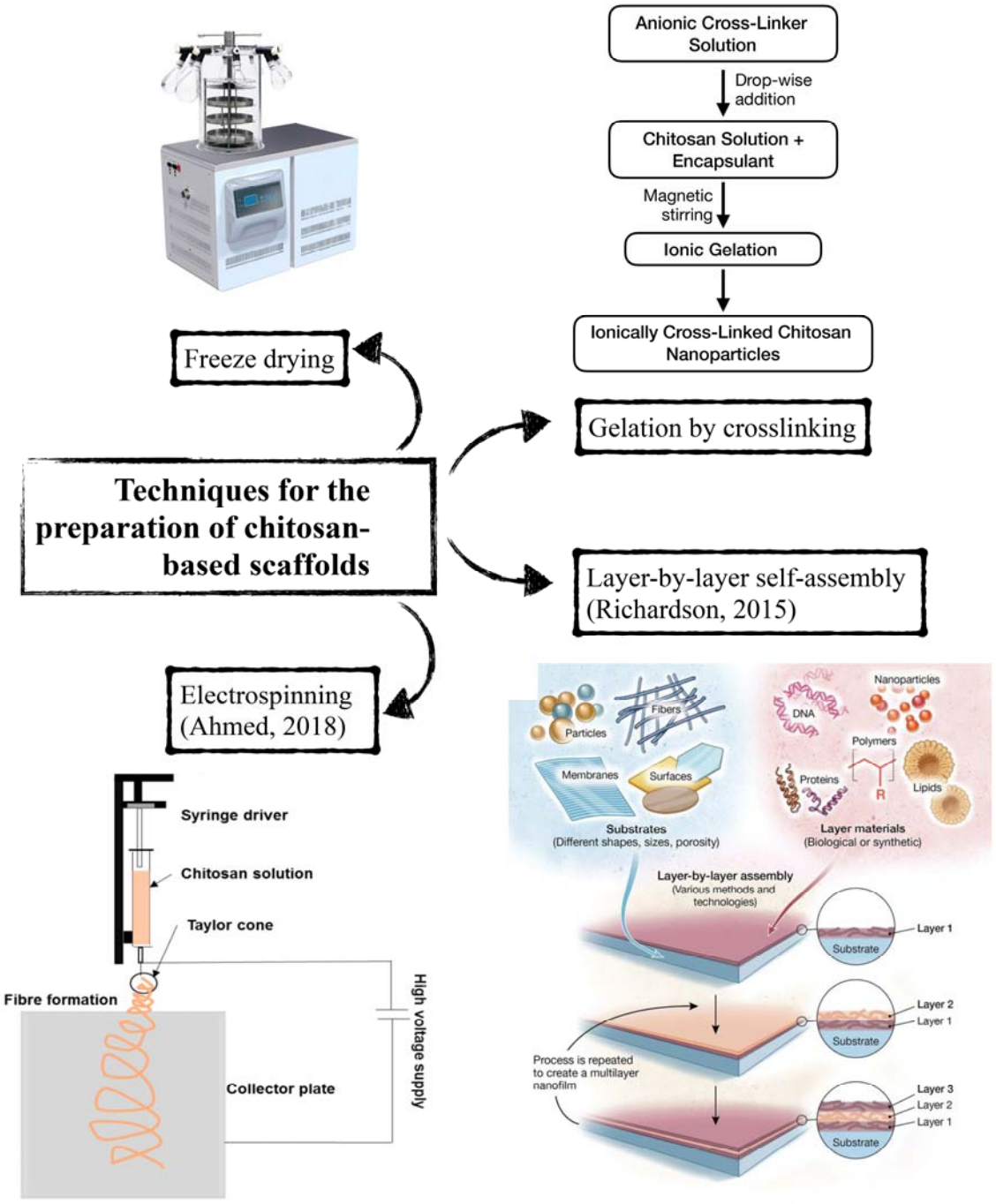

Figure 3. Techniques for the preparation of chitosan-based scaffolds. Adapted with permission from Refs. [61,72]. [61], Copyright 2018, Elsevier. [72], Copyright 2015, Science.

The deposition of inorganic mineral ions on organic CS templates generally involves classical and nonclassical crystallization. Classical crystallization indicates that a new phase appears through the association of monomeric units (e.g., molecules, atoms, or ions). Nonclassical crystallization is based on dynamic mechanisms, implying that crystallization can occur by the attachment of a wide range of species more complex than simple ions. The higher-order species, including multi-ion complexes, oligomers (or clusters), and nanoparticles crystalline, amorphous, or liquid, are formed as intermediates between dispersed particles and true single crystals [73]. Most of the direct ion reaction methods, including the wet chemical method (WCM) [74], simulated body fluid soaking method (SBF) [75], and solution-gelatin method [76], are based on classical crystallization. WCM

is one of the most commonly used methods for mineralized CS scaffolds. Briefly, the solution containing calcium and phosphorus ions is directly mixed with and soaked in a CS template to allow the deposition of calcium and phosphorus ions. The simulated body fluid soaking method refers to soaking CS into an SBF solution for some time to deposit bone-like minerals on the CS template [77]. Conventional SBF is a solution containing Ca^{2+}, HPO_4^{2-}, and other components, whose ionic concentrations and pH values are similar to those of human blood plasma [78]. The sol-gel method has been developed recently for synthesizing organic/inorganic hybrid polymers. Fine metal oxide particles (SiO_2 and TiO_2) are compounded into organic polymers through the hydrolysis and condensation of alkoxy metal organic compounds [M $(OR)_4$] to obtain organic/inorganic hybrids with special properties [79]. The polymer-induced liquid precursor (PILP) method [80] and alkaline phosphatase (ALP) enzyme-induced method [81] are mineralization methods based on nonclassical crystallization. In the PILP method, calcium carbonate is formed by the deposition of a liquid-phase mineral precursor [80]. The addition of acidic polypeptide leads to liquid–liquid phase separation of precursor droplets, and thus, the droplets accumulate on the surface, formulating the films, dehydrating to transform from amorphous to crystalline [82]. The ALP method involves CS scaffolds that were pre-immersed in ALP solution, then transferred to phosphate solution, where ALP cleaves phosphate ions from ß-GP and reacts with calcium ions to realize mineralization [83].

4. Applications of Mineralized CS Scaffolds in BTE

Considering the good biocompatibility and stable properties of CS, various components have been added to mineralized CS scaffolds in recent years to further enhance osteogenesis. The scaffolds are divided into three categories based on different components that are combined with CS scaffolds: (i) pure mineralized CS scaffolds, (ii) mineralized CS scaffolds without other organic components, and (iii) mineralized CS scaffolds with other organic components.

4.1. Pure Mineralized CS Scaffolds

The important properties of different mineralized CS scaffolds without other components (such as organic, inorganic, biological factors, and so on) in the field of BTE are presented in Table 1. Baskar et al. fabricated CMC-HA nanoparticle composite scaffolds. The results showed that a 1:5 HA–CMC *w*/*w*% concentration resulted in improved bioactivity, cell viability, and proliferation, in addition to enhanced expression of dentin sialophosphoprotein, and vascular endothelial growth factor mineralization markers when tested in vitro [84]. Some scholars modified inorganic minerals and prepared corresponding mineralized CS scaffolds. Chen et al. combined a facile strategy technology with a biomimetic mineralization process, preparing a superoleophobic film. Chitosan modified with methacrylic anhydride (CSMA) is prepared by acylation as the scaffold to spread a layer of calcium carbonate. Due to the uniform structure of the surface, the film has higher transparency and mechanical properties than general oil-repellent film [85]. Jindal et al. fabricated mesoporous zinc silicate-fortified CS scaffolds (mZS–CS scaffolds); 0.3 wt% of mZS loading composite scaffolds showed good biocompatibility and no obvious toxicity. The addition of mZS also improved the antibacterial activity of scaffolds [86].

Table 1. Applications of different mineralized chitosan scaffolds without other components in BTE.

Chitosan or Its Derivatives	Minerals	Important Conclusions	Reference
Chitosan	HA	The scaffolds promoted osteogenic differentiation of pre-osteoblasts in vitro and demonstrated excellent tissue integration in vivo.	[83]
Carboxymethyl chitosan (CMC)	HA	Human dental pulp stem cells (hDPSCs) on 1:5 HA-CMC scaffolds displayed increased cell viability/proliferation and enhanced DSPP as well as VEGF expressions.	[84]

Table 1. *Cont.*

Chitosan or Its Derivatives	Minerals	Important Conclusions	Reference
Chitosan	Mesoporous zinc silicate (mZS)	The 0.3 wt% of mZS loading composite scaffolds showed good biocompatibility and no obvious toxicity. Addition of mZS also improved the antibacterial activity of scaffolds.	[86]
Chitosan	HA	75/25 *w*/*w* HA/CS scaffolds provided an effective space for new bone formation.	[87]
Water-soluble phosphate functionalized chitosan (CSMAP)	Strontium phosphosilicate (SPS)	The bioactive Sr, P and silicon were released from CSMAP-SPS hydrogels in a sustained and controlled manner at a non-toxic level.	[88]
Chitosan	$LaPO_4$; β-TCP	The scaffolds showed no obvious toxicity or effects on cell morphology, and they accelerated bone generation in a rat cranial defect model.	[89]
Chitosan	Zinc-containing nanoparticle-decorated ultralong hydroxyapatite nanowires (Zn-UHANWs)	The scaffold can enhance the osteogenic differentiation of rBMSCs and facilitate new bone formation in the bone defect region.	[90]
Chitosan	HA; Whitlockite (WH)	The WH-CS scaffolds had a better biocompatibility, enhancing proliferation and osteogenic differentiation ability of human bone mesenchymal stem cells (hBMSCs) than HA/CS scaffolds.	[91]

4.2. Mineralized CS Scaffolds without Other Organic Components

The important properties of scaffolds with other inorganic components in the field of BTE are depicted in Table 2. CS is the only organic template; different inorganic components or bioactive molecules constitute the scaffolds with CS. Gritsch et al. combined copper and strontium into HA–CS scaffolds [92]. The results showed that copper and strontium exhibited different release rates in the scaffold. In the initial stage of material implantation, copper was released explosively to achieve an antibacterial effect, while strontium was released slowly with material degradation to promote bone repair (Figure 4). Balagangadharan et al. fabricated HA–CS–$nZrO_2$ biocomposite scaffolds [93]. The scaffolds showed osteoinductive properties, and adding bioactive molecules, such as miR-590-5p (a kind of microRNA), to the scaffolds further enhanced osteoblast differentiation. PLGA nanospheres were used to load bioactive factors rhBMP-2 and p24 and combined with HA–CS scaffolds. The results showed that the composite vehicle had good biocompatibility and osteoinduction. Yang et al. fabricated magnetic mesoporous calcium silicate–CS (MCSC) scaffolds, which were made of M-type ferrite particles ($SrFe_{12}O_{19}$), mesoporous calcium silicate ($CaSiO_3$), and CS [94]. The MCSC loaded with doxorubicin (DOX) showed robust antitumor and bone regeneration properties under photothermal therapy (PTT). Among the methods, the $CaSiO_3$ microspheres were used to enhance drug delivery, and the $SrFe_{12}O_{19}$ particles were used to improve the efficiency of PTT. Scaffolds containing CS, calcium polyphosphate (CaPP), and pigeonite (Pg) particles was prepared for bone regeneration. The results showed that CS/CaPP scaffolds containing Pg particles at 0.25% concentration enhanced the proliferation and osteoblast differentiation of mouse mesenchymal stem cells in vitro [95]. Yildizbakan et al. prepared a controlled release of antibacterial agents from CS scaffolds combined with iron-doped dicalcium phosphate dihydrate (Fe-DCPD) minerals and cerium oxide nanoparticles. All scaffolds exhibited inhibitory effects on bacterial growth against Staphylococcus aureus and Escherichia coli strains [96].

Table 2. Applications of mineralized scaffolds which chitosan is the only organic template in BTE.

Chitosan or Its Derivatives	Minerals	Other Inorganic Componets	Bioactive Molecule	Important Conclusions	Reference
Chitosan	HA	Strontium; Copper	None	The release of copper and strontium followed significantly different profiles due to the different nature of the loading.	[92]
Chitosan	HA	Nano-zirconium dioxide ($nZrO_2$)	MicroRNA (miRNA)-miR-590-5p	CS/HA/nZrO2 scaffolds promoted osteoblast differentiation, and this effect was further increased in the presence of miR-590-5p in C3H10T1/2 cells.	[93]

Table 2. *Cont.*

Chitosan or Its Derivatives	Minerals	Other Inorganic Componets	Bioactive Molecule	Important Conclusions	Reference
Chitosan	Mesoporous calcium silicate (MCS)	$SrFe_{12}O_{19}$ particles	Doxorubicin (DOX)	The MCS scaffolds possessed the excellent anti-tumor efficacy via the synergetic effect of DOX drug release and hyperthermia ablation.	[94]
Chitosan	Pigeonite (Pg)	Calcium polyphosphate (CaPP)	None	The inclusion of iron-containing Pg particles at 0.25% concentration in CS/CaPP scaffolds showed enhanced bioactivity by protein adsorption and biomineralization, compared with that shown by CS/CaPP scaffolds alone.	[95]
Chitosan	Silica	ZnO	Mangiferin	With the higher amount of nano-ZnO, the compressive strength and modulus increased.	[97]
Chitosan	HA	None	p24; rhBMP-2	This research showed that the composite vehicle could exhibit sustained release of osteogenic factors.	[98]
Chitosan	HA	None	Bone Morphogenetic Protein (BMP-2)	The BMP-2-TAK1-p38-OSX signaling pathway may play an important role in bone repair mediated by rhBMP-2 loaded hollow HA microspheres/CS composite.	[99]
Chitosan	Mesoporous calcium silicate (MCS)	Gadolinium (Gd)	None	The scaffolds supported the cell spreading and proliferation, and stimulated the new bone in-growth toward scaffold interiors.	[100]
Chitosan	Calcium phosphate cement (CPC)	Iron oxide nanoparticles (IONP)	None	CPC with 3% IONP doubled its flexural strength and had the greatest promotion of osteogenic differentiation of the stem cells.	[101]
Chitosan	Silicoalumino phosphates (SAPO-34)	Fe; Ca	None	Fe-Ca-SAPO-34/CS scaffold possessed excellent cytocompatibility, and supported the adhesion, spreading, and proliferation of cells.	[102]

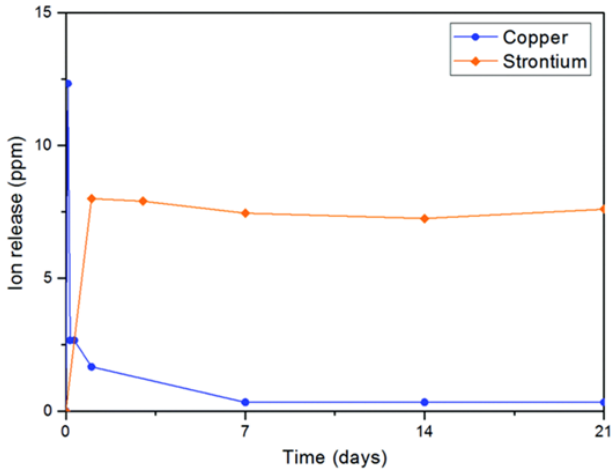

Figure 4. Release of copper from copper–chitosan and of strontium from Sr–HA particles: the difference in release can be clearly observed. While copper is characterized by burst release, the release of strontium is uniform over time. Adapted with permission from Ref. [92]. Copyright 2019, Royal Society of Chemistry.

4.3. Mineralized CS Scaffolds with Other Organic Components

Nowadays, researchers also combine CS with other polymers to prepare multi-organic-component mineralized scaffolds. The important properties of these scaffolds in the field of BTE are presented in Table 3. Dasgupta et al. compared the efficacy of gelatin-CS-based bone scaffolds after incorporating three different bioactive nanoparticles, including HA, β-TCP, and 58S BG (58BG), by evaluating its physicochemical, mechanical, and osteogenic properties [103]. The results demonstrated that the prepared scaffolds (30 wt% 58BG) might serve as better bone substitute materials because of their higher bioactivity in bone tissue regeneration. Zhao et al. evaluated the bone regeneration potential of the nanohydroxyapatite/CS/gelatin (HA–CG) three-dimensional porous scaffolds with transplanted human periodontal ligament stem cells (hPDLSCs) [15]. The results showed that the hPDLSCs adhered well to the scaffolds and significantly improved the bone formation. Other than, Col and gelatin, many other polymers such as PLGA [104] and silk fibroin [105] were also

applied in the CS scaffolds as components. Deng et al. prepared a temperature-sensitive chitosan hydrogel loaded with rhBMP-2 using chitosan and β-glycerophosphate, which could effectively control the early burst release of rhBMP-2. After the outer layer of the chitosan scaffold was slowly degraded, rhBMP-2 microspheres in the scaffold were slowly and steadily released. Sustained-release administration ensures long-term activity of rhBMP-2, and the pattern is more similar to that of cytokine release in vivo, allowing better formation of bone tissue [104]. Ji et al. prepared hydroxyapatite/tannic acid/chitosan/sodium alginate scaffolds with different curcumin-loaded silica microspheres by 3D printing for bone regeneration. The scaffolds had high biomineralization capability and a good degradation rate, which can enhance the proliferation of bone marrow mesenchymal stem cells significantly [106]. Cross-linking agents may affect the biological properties of the CS-based scaffolds. Using ammonium hydroxide to generate physical entanglements and prepare scaffolds had been proved to be non-cytotoxic [107]. Bioglass/chitosan/alginate (BCA) composite scaffolds prepared by the freeze-drying method have potential applications in BTE. With the adding alginate of BCA, the mechanical strength of BCA and the mineralization ability of Bioglass were effectively enhanced [108]. Chitosan scaffolds incorporated with calcium hydroxide (CH-Ca) and simvastatin (SV) promoted an increase in bioactivity [109].

Table 3. Applications of multi-organic-component mineralized scaffolds in BTE.

Chitosan or Its Derivatives	Minerals	Other Organic Componets	Other Inorganic Componets	Bioactive Molecule	Important Conclusions	Reference
Carboxymethyl chitosan (CMC)	HA	Collagen (Col)	None	None	Synergistic mineralization can increase the mechanical strength and decrease the degradation rate of collagen scaffolds at the same time such that the BMC scaffolds can better promote the regeneration of bone tissue in defects.	[38]
Chitosan	HA; β-TCP; BG	Gelatin	None	None	The gelatin-chitosan scaffold with 30 wt% of synthesized 58S bioactive glass (GCB30) showed higher capacity to proliferate MSCs cultured onto it as compared to other composite scaffolds.	[103]
Chitosan	HA	Polylactic-coglycolic acid (PLGA)	None	Recombinant human bone morphogenetic protein 2 (rhBMP-2)	PLGA/HA/CS/rhBMP-2 scaffold complex effectively controlled the early burst effect of rhBMP-2.	[104]
Chitosan	HA	Polyvinyl-alcohol (PVA)	None	Platelet-rich plasma (PRP); Mesenchymal stem cells (MSCs)	The in vivo results demonstrated that in the animals implanted with PVA-chitosan-HA, the defect was repaired to a good extent, but in those animals that received MSCs-seeded PVA-chitosan-HA, the defects were almost filled.	[110]
Chitosan	BG	Vanillin	None	None	The 3D porous chitosan-vanillin-BG (CVB) scaffold had improved mechanical properties, anti-microbial ability, and osteoconductivity.	[111]
Chitosan	HA	Phoenix dactylifera seeds (PD)	None	None	The PD-CS scaffold is a potential candidate to promote osteoblast cell growth and osteogenic differentiation.	[112]
Chitosan	Halloysite nanotubes (mHNTs): aluminosilicate	β-Glycerophosphate (GP)	None	Icariin (IC)	IC/mHNTs led to the improved mechanical strength of chitosan hydrogel and enhanced differentiation of encapsulated human adipose-derived stem cells (hASCs) into bone tissue.	[113]
Chitosan	CuMn-HA	Polyvinyl pyrrolidone (PVD)	None	None	10, 20, 30 wt% of CuMn-CS-HA biocomposite exhibited great material characteristics where 30% (BC-3) displayed the minimum swelling. BC-3 has improved mechanical properties, physiochemical characteristics and apatiteforming capabilities.	[114]
Chitosan	Zeolitic imidazolate framework-8 nanoparticle (ZIF-8)	Catechol(CA)	None	None	The 30 mg/1.2 mg CA/ZIF-8 hydrogel and bone powders showed the largest new bone formation area and thickness.	[115]

Table 3. *Cont.*

Chitosan or Its Derivatives	Minerals	Other Organic Componets	Other Inorganic Componets	Bioactive Molecule	Important Conclusions	Reference
Hydroxyethyl chittosan (HECS)	BCP	Polyvinyl alcohol(PVA)	None	None	The reinforced HECS/PVA/BCP hydrogel with promising mechanical and biological properties has the potential for application in bone regeneration.	[116]
Chitosan	Halloysite (HAL)	Alkaline phosphatase (ALP); collagen	None	None	Mineral was formed in both CS and Collagen-CS scaffolds with HAL-ALP, the process was more effective for collagen-containing hydrogels. Collagen-CS scaffolds containing 30% of HAL-ALP have the highest potential as bioactive material for bone regeneration.	[117]
Lactose-modified chitosan (CTL)	HA	Alginate	None	None	The scaffolds showed remarkable stability up to 60 days of aging. CTL-coating did not affect cell proliferation, but stimulated cell differentiation.	[118]
Chitosan	HA	Poly (lactic acid)	Au; Pt; TiO_2	None	The highest bioactivity in contact with cells exhibited samples modified with HA and amorphous titanium dioxide NPs, while scaffolds containing nanogold showed highest positive impact on DC-stimulated in vitro biomineralization.	[119]
Chitosan	BG	Chondroitin sulfate	None	None	The scaffold facilitates enhanced ALP activity, biomineralization and collagen type I expression of cells and thereby chitosan/chondroitin sulfate/BG might be a suitable candidate for bone tissue engineering.	[120]
Chitosan	Calcined diatomite; Polyhedral oligomeric silsesquioxanes (POSS); Si-HA	Na-carboxymethyl cellulose (Na-CMC)	None	None	All inorganic reinforcements increased the mechanical strength, enhanced the water uptake capacity and fastened the degradation rate. The nanocomposite scaffolds did not show any cytotoxic effect and enhanced the surface mineralization in osteogenic medium.	[121]
Chitosan	HA	Collagen (Col)	Functionalized multiwalled carbon nanotube	None	The Col/f-MWCNT/CS scaffolds had higher in vitro bioactivity, large surface area, and a good pore volume, interconnected porous microstructure.	[122]
Carboxymethyl chitosan (CMC)	HA	Poly(dopamine) (PDA)	None	None	HA/PDA/CMC composite scaffolds could promote more osteogenic differentiation of mouse bone marrow stromal cells (mBMSCs) than scaffolds without PDA in vitro and the effect was not hindered by the photothermal process.	[123]
Chitosan	HA	Ursolic acid	None	None	The HA-CS-UA scaffolds had good anti-inflammatory, osseointegration, osteo-inductivity, and bone regeneration.	[124]

Although numerous mineralized CS scaffolds with low biotoxicity and good bioosteogenic effects had been developed in recent years, a lack of standardized study protocols and approaches make it a challenge to compare different study outcomes and promote the further development of mineralized CS scaffolds for BTE. The composition and preparation methods of the composite scaffolds will have different effects on the mechanical properties, biotoxicity, degradability, and bone promotion of the scaffolds. At the same time, there is still a long way to go for mineralized chitosan scaffolds from bench to bed, and the clinical study results of different mineralized chitosan scaffolds in the future are exciting.

5. Conclusions

A review of the literature revealed that a large number of researchers combined mineralized CS scaffolds with other components (organic, inorganic, and biological factors) to develop a variety of scaffolds for BTE; most of these scaffolds were proven to be osteoconductive and osteoinductive. Researchers can prepare scaffolds, provide more powerful means for bone regeneration, and investigate the mechanism of bone regeneration with the development of manufacturing technology. However, the existing scaffolds have some defects: (i) Bone healing is a phased, dynamic, and orderly process. The simple composites of scaffolds cannot adapt to different stages of bone healing. At present, although a variety of composite scaffolds have been developed, intelligent and controllable scaffolds

still need to be further explored. Future research should aim at safe and effective bone regeneration by realizing a highly controllable drug sustained release system or regulating the release of organic, inorganic, and growth factors in an organized manner. (ii) The existing scaffolds are in the primary research stage, with few scaffolds that can be used in a clinical setting. The effect of scaffolds on the human body needs further discussion. (iii) Recent studies show numerous mineralized CS scaffolds with good osteogenic effects. However, researchers have not standardized their experimental study protocols and have employed different approaches for characterizing scaffolds and reporting their findings. Hence, comparing different study outcomes is extremely difficult. Moreover, the lack of further research on the mechanism of osteogenesis may be the direction that the researchers should explore in the future.

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