11 Chitosan-Based Biocomposites for Biomedical Application *Opportunity and Challenge*

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CONTENTS

11.1 INTRODUCTION

Many polymers have been used for biomedical applications. The polymers include synthetic polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly (lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL), poly(ethylene glycol) (PEG),

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polyurethane, poly(vinyl alcohol) (PVA), and natural polymers like alginate (AL), gelatin (GEL), starch (ST), collagen (COL), and chitosan (CS) [1]. Among them, natural polymers are especially interested in biomedical uses because their biological and chemical properties are similar to natural tissues due to the natural components of living structures [2]. Among naturally derived polymers, CS has been much attracted in biomedical applications because it has unique biological properties such as biocompatibility, biodegradability, nontoxicity, antibacterial, and anti-fungistatic properties [1].

The CS can be obtained from the source of chitin as one of the most abundant materials being second only to cellulose (CEL) produced annually by biosynthesis. The chitin is an important component of the exoskeleton in animals and the principal fibrillar polymer in the cell wall of fungi [3]. The CS as one of the linear polysaccharides is composed of glucosamine and *N*-acetyl glucosamine residues linked via β(1,4) glycosidic bonds. The content of glucosamine should be over 60 mol.% to be called the CS after deacetylation from the chitin named as the degree of deacetylation (DD) although the molecular weight of the CS ranges from 300 to over 1,000 kD with a DD from 30% to 95% according to the source and preparation procedure [4]. The chemical structure of the CS provides many possibilities for ionic and covalent modifications to allow extensive adjustment of physicochemical and biological properties because the CS has three kinds of reactive functional groups such as amino group as well as both primary and secondary hydroxyl groups [1]. The CS has various biomedical applications in wound dressing [5,6], tissue engineering [7,8], and drug delivery carriers [9,10] because it provides several advantages of easily processed into sponge-like forms [11], scaffolds [12], microparticles [13], nanoparticles (NPs) [14], nanofibers [15], beads [16], and membranes [17].

Much attention has been focused on the use of CS and CS derivatives in biomedical applications over the last few decades. However, it is very difficult to make any available products using them due to several limitations such as insolubility in neutral pH, brittleness, weak mechanical property, and instability at environmental conditions [18]. In this review, we are aimed to overcome the limitations of CS alone for wound dressing and bone tissue engineering applications using CS-based biocomposites.

11.2 REQUIREMENT OF WOUND DRESSINGS

Wound healing is a complex and regulated physiological process that involves the activation of various cell types through several subsequent such as homeostasis, inflammation, proliferation, and tissue remodeling [19]. Therefore, it is important to use well-designed wound dressings to meet the wound healing cascade for ensuring optimal healing. There are several key parameters to design the optimal wound dressing. First, a moist wound environment for the wound dressing is very important because it facilitates the recruitment of immune cells to promote wound healing by the elaboration of several growth factors and it decreases pain during dressing changes [20]. Second, absorption of excess exudates and blood at the wound site is very critical for the wound dressing because the excess exudates contain degrading enzymes in the tissue which affect the activity and proliferation of cells with losing function of growth factors thus delay of the wound healing process [21]. Third,

TABLE 11.1
The Requirement of Wound Dressings

Requirement	Ref.
Moist wound environment for recruitment of immune cells and decrease of pain	[20]
Absorption of excess exudates and blood at the wound site for removing degraded enzymes	[21]
Prevention of infection and protection of bacterial invasion for favorable host repair	
Adequate water and oxygen exchange for cell metabolism	[22]
Low adherence for prevention of trauma and pain for easy removal	[20]
Acceptable mechanical properties for topical application and controllable degradation	[23]
Should not induce a toxic or inflammatory response	
Cost-effective and minimal frequency of wound dressing change	

prevention of infection and protection of bacterial invasion should be controlled because the infected wound gives an unpleasant odor, delays extracellular matrix synthesis, and prolongs the inflammatory phase which results in further microbial contamination to the tissue. Theoretically, wound dressings should be acted as a barrier between the wound area and the outside environment. Fourth, adequate water and oxygen exchange should be controlled because oxygen is one of the essential nutrients for cell metabolism, and exudates can be managed by the permeability of wound dressings to the water vapor [22]. Fifth, the wound dressings should be low adherent to the wound site for prevention of trauma, and pain because the strongly adherent wound dressings induce further tissue damage at the wound site and should be easy on removal. Sixth, mechanical properties should be acceptable to be compatible for topical application of wound dressings and degradation should be adjusted to match the timeline with the healing process. Seventh, the wound materials should not induce a toxic or inflammatory response. Finally, the wound dressings should be cost-effective and minimal frequency of change in terms of economic condition. The desirable requirement of wound dressings is summarized in Table 11.1.

11.3 CHITOSAN-BASED BIOCOMPOSITES FOR WOUND DRESSING APPLICATION

In this section, we discuss CS-based biocomposites to meet the requirement of wound dressings mentioned in the previous section. Among the several requirements, we focus on how to increase the mechanical property, how to prevent bacterial invasion, how to get anti-inflammation and antioxidants, how to keep water absorption, and how to have a multifunction property in the CS-based biocomposites for the application of wound dressing.

11.3.1 CS-Based Biocomposites Having Acceptable Mechanical Properties

Acceptable mechanical properties for the application of wound dressings should be compatible according to the physical forms such as film, lint, gauze, hydrocolloid, hydrogel, and skin scaffold [24].

CS films have poor mechanical properties which limit their applications for wound dressings. The CS-based biocomposites should be used to improve mechanical properties. Among CS-based biocomposites, CEL has been widely used to blend with the CS [25] because it is the most abundant and renewable polymer available in the world [26]. However, it is insoluble in water and general organic solvents due to the intermolecular hydrogen bonding among hydroxyl groups of the β-D-glucopyranose unit. Many types of research have been tried to achieve the solubility of CEL. Different CEL derivatives have been used to solve the solubility of the CEL by chemical substitution. Among them, hypromellose succinate (HPMCS) obtained by grafting of succinic and with hypromellose was prepared by Jiang et al. [27] because the HPMCS has a good film-forming ability due to the free carboxyl acid groups after dissolving in water. And then, the HPMCS-CS hydrogel films were prepared by amide bond formation among carboxyl acid groups in the HPMCS and amino groups in the CS using 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as a condensing agent and were assessed the applicability of the hydrogel films as a wound dressing. The results indicated that the mechanical properties of HPMCS-CS hydrogel films were significantly increased both in the dry and swollen state due to the crosslinking between HPMCS and CS compared with those of HPMCS/CS blend film, a suggestion of potential wound dressing although it is not cost-effective due to the several steps to prepare the hydrogel films.

Cho's group prepared the semi-interpenetrating polymer network (semi-IPNs) composed of CS and to enhance the mechanical properties of CS because the brittleness of the CS itself limits the wound dressing application [28]. The results indicated that the formation of Semi-IPNs between CS and poloxamer as shown in Figure 11.1 [28], the mechanical strength of semi-IPNs sponge remarkably increased due to the intermolecular hydrogen bonding between CS and poloxamer compared with CS/poloxamer blend. Also, they evaluated wound healing in a mouse skin defect model using CS/poloxamer semi-IPNs [29]. The results indicated that the wounds covered with CS/poloxamer semi-IPNs were filled with new epithelium without any adverse reactions, an indication of potential wound dressing biocomposites.

Akhavan-Kharazian et al. prepared and characterized CS/GEL)/nanocrystalline CEL (NCC)/calcium peroxide (CP) films to improve the mechanical properties of CS itself for potential wound dressing applications [30]. The results indicated that CS-based biocomposites with the combination of CP and NCC improved the hydrogen bonding between functional groups than CS itself with the antibacterial activity against E *coli* although the addition of CP and NCC particles reduced the amount of water vapor transmission rate and swelling, a suggestion of potential wound dressing materials.

Gao et al. prepared and characterized minocycline (MIN)-loaded carboxymethyl CS (CM-CS) gel/AL nonwoven biocomposites to overcome weak mechanical properties of CM-CS gel by coating MIN/CM-CS on the surface of plasma-treated calcium AL fiber needle-punched nonwovens and were crosslinked with EDC/NHS for wound dressing applications [31]. The wound dressing increased the mechanical properties of CM-CS itself due to the crosslinking and provided quickly absorbed wound exudates with the anti-bacterial property due to the porous biocomposite structure, a suggestion of a new functional wound dressing although it is very

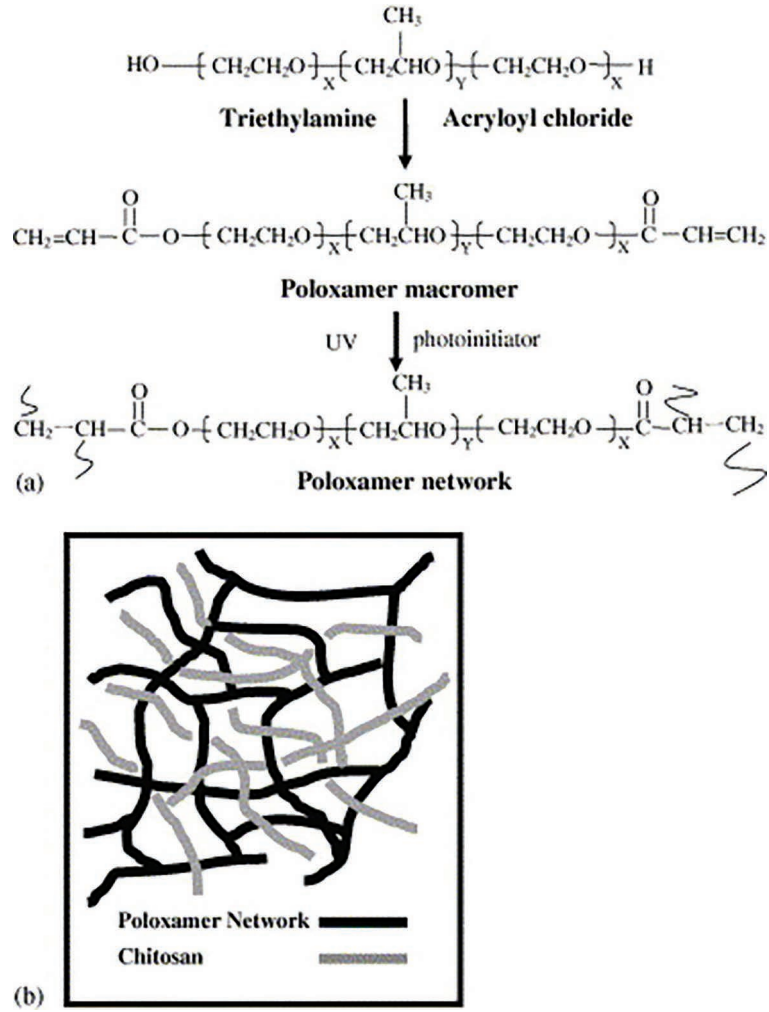


FIGURE 11.1 Scheme of poloxamer macromer and poloxamer networks from poloxamer macromer (a), and structure of CS/poloxamer semi-IPNs (b).

difficult to apply for the clinical healing of wounds due to the several steps of the preparation.

Patholamuthu et al. prepared CS-based electrospun biocomposites composed of CS, poly(ethylene oxide), and aloe vera to get mechanically strong enough to endure mechanical stimulus and to induce wound healing in *vivo* [32]. The results indicated that the mechanical properties of the biocomposite measured by a novel spirograph-based mechanical system (SBMS) were improved compared with those prepared by the static system due to the preparation of the uniformity electrospun mat by the SBMS, a suggestion of the importance of uniformity in the mechanical properties of the electrospun mat for wound dressing application.

Rahmani et al. prepared CS-based biocomposites composed of CS, PVA, and poly(vinyl pyrrolidone) (PVP) using 1,6-diamino carboxy sulfonate) (HMDACS) as a cross-linking agent for wound dressing agents [33]. The results indicated that the mechanical properties of the biocomposites depended on formulations of biocomposite films having the highest mechanical properties when formulated with CS (50 wt%), PVA (30 wt%), PVP (20 wt%), and HMDACS (2 wt%) although the best antibacterial activity against E *coli* was found in different formulation.

Khalili et al. prepared CS-based biocomposites containing CS, poly (phenyl sulfide) (PPS), and reduced graphene oxide (GO) for wound dressing application because the PPS has high mechanical properties due to the semi-crystalline polymer and the GO enhance the cellular activity [34]. The results indicated that the stress shown in Figure 11.6A [34] and compressive modulus shown in Figure 11.6B [34] were enhanced with the PPS/GO addition to the CS although the nonlinear behavior of the compression modulus in the biocomposites was found due to the water molecule present in the hydrogel structure [35].

11.3.2 CS-Based Biocomposites Having Antibacterial Properties

The wound dressings should have antibacterial properties because they can be used as the topical application in the outside microenvironment and gross microbial contamination delays the wound healing.

Diez-Pascual et al. prepared biocomposites containing castor oil (CO) polymeric films mixed with CS-modified ZnO nanoparticles (NPs) as shown in Figure 11.2 [36] to get antibacterial properties for the application of wound dressings [36] because the ZnO has antibacterial activity against both Gram-positive and Gram-negative bacteria even in the absence of light [37].

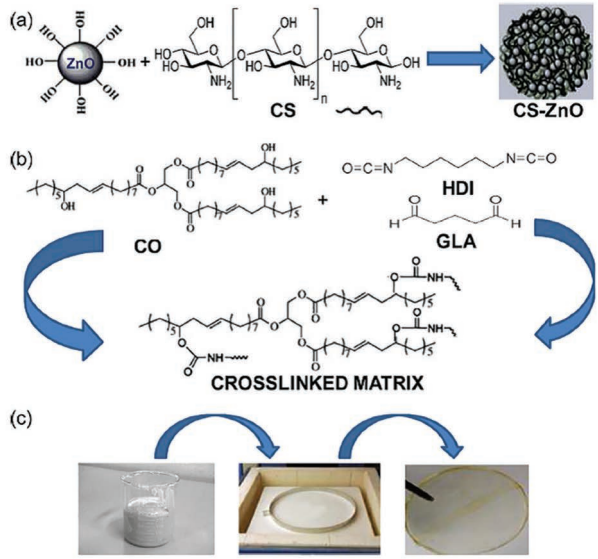
The results indicated that the antibacterial activity of the films against bacteria was increased with an increase of CS-ZnO content although the biocomposite films showed cytotoxicity *in vitro* when CS-ZnO NPs were mixed over 0.5wt%.

Amalraj et al. prepared PVA/gum Arabic (GA)/CS biocomposite films contained with black pepper essential oil (BPEO) and ginger essential (GEO) to have antibacterial activity for the wound dressing application [38] because both BPEO and GEO have strong antibacterial activity [39]. The results indicated that the BPEO/GEO-loaded PVA/GA/CS films significantly inhibited the growth of Gram-positive and Gram-negative bacteria, an indication of promising wound dressing and food packaging materials.

Haider et al. fabricated CEL-based biocomposite comprising adsorbed CS and silver (Ag)NPs to get the antibacterial property for application as wound dressings [40] because the AgNPs have an effective anti-microbial property [41]. The results indicated that AgNPs-loaded CS/CEL biocomposites exhibited good antibacterial activity against both Gram-positive and Gram-negative bacterial strains, an indication of the potential application of the wound dressing agent.

Sathiyaseelan et al. prepared CS-based biocomposites containing fungal CS (FCS), aloe vera extract (ALE), and Cuscuta reflexa-mediated AgNPs (CUS-AgNPs) to get the antibacterial property for wound dressing application because the CUS-AgNPs have less toxicity than general AgNPS [42]. The results indicated that CUS-AgNPs-loaded

Scheme 1[a]



[a](a) Structure of CS and representation of CS-ZnO NPs; (b) schematic representation of CO, HDI, GLA and the crosslinked matrix; (c) depiction of the film casting process.

FIGURE 11.2 (a) Structure of CS and representation of CS-ZnO NPs. (b) Schematic representation of CO, HDI, GLA, and the cross-linked matrix. (c) Depiction of the film casting process.

FCS/ALE sponges showed higher antibacterial activity against Gram-positive and Gram-negative bacteria compared with other groups without CUS-AgNPs due to the antibacterial property of the CUS-AgNPs, suggestion of potential wound dressing.

Cahu et al. prepared and evaluated CS-based biocomposite films containing GEL, chondroitin-4-sulfate (C4S), and ZnO NPs for wound dressing application [43] because the ZnO NPs have antibacterial and anti-inflammatory properties [44]. The results indicated that the CS/C4S/GEL/ZnO NPs films highly inhibited the growth of staphylococcus aureus compared with other groups and significantly enhanced wound contraction of rat skin with full-thickness when compared with other groups after 6 days, an indication of potential wound dressing application.

Sergi et al. prepared CS-based biocomposites composed of CS and several metal-containing bioactive glass gauges for wound dressing application [45] because the metal-containing bioactive glasses have antimicrobial activity [46]. The results indicated that CS-based composites containing bioactive glass gauge showed enhanced cell adhesion and proliferation and wound healing compared with CS itself due to the release of Sr, Mg, and Zn ions from the bioactive glass although they did not check the antibacterial property.

Xia et al. prepared CS-based biocomposites containing quaternary ammonium CS NPs, and CS to have asymmetric wettable surfaces [47] because asymmetric property enables the CS-based biocomposites to have a hydrophobic outer surface for showing waterproof and antiadhesion contamination properties and to have a

hydrophilic inner surface for preserving water-absorbing ability. The results indicated that the QACS NPs/CS biocomposites promoted wound healing and angiogenesis with the effective prevention of wound infection, an indication of a promising dressing biomaterial for chronic wounds.

11.3.3 CS-Based Biocomposites Having Anti-inflammation and Antioxidant

In severe pathological conditions during wound healing, the general cascade of the wound healing process is lost and the wounds are locked in chronic inflammation with abundant neutrophil infiltration and release of reactive oxygen species (ROS) and reactive nitrogen species [48]. Therefore, anti-inflammatory and antioxidant should be used to mitigate the deregulated chronic inflammation during wound healing.

Negi et al. prepared thymoquinone (TQ)-loaded CS-lecithin NPs to incorporate them into Carbopol hydrogel for the wound dressing application [49] because the TQ has anti-inflammatory and antioxidant properties [50]. The results indicated that the Carbopol hydrogel incorporated with TQ-loaded CS-lecithin NPs exhibited superior wound healing efficacy and wound reduction in wound model mice compared with TQ or silver sulfadiazine although they did not check the anti-inflammatory and antioxidant properties.

Ehterami et al. prepared CS/AL hydrogels containing vitamin E (V-E) for wound dressing application in a rat model [51] because the V-E has been known as an antioxidant by protecting cell membranes from ROS attack [52]. The results showed that V-E-loaded CS/AL hydrogel-based biocomposites had a higher wound contraction than the gauge-treated wound as the control, a suggestion of potential wound dressing material.

Augustine et al. prepared electrospun PCL membranes containing CS ascorbate for wound dressing application [53] because the CS ascorbate promoted periodontal regeneration due to the reduced migration of inflammatory cells [54]. The results indicated that CS ascorbate-loaded electrospun membranes showed better cell adhesion and cell viability than PCL membranes although they did not perform wound healing properties.

Zhu et al. prepared CS-based electrospun biocomposites containing asiaticoside (AS), AL, PVA, and CS to evaluate the healing effect on deep partial-thickness rat burn injury [55] because the AS has anti-inflammatory and antioxidant activities [56]. The results indicated that wound healing on deep partial-thickness burn injury of a rat was significantly improved by the AS-loaded AL/PVA/CS electrospun nanofibers due to the downregulation of tumor necrosis factor and interleukin-6 by the loaded AS.

11.3.4 CS-Based Biocomposites Having Multifunctional Properties

Wound healing is a complex and dynamic process by the defense mechanism of the body through the wound healing cascade for ensuring optical healing [21]. Therefore, multifunctional properties such as biocompatibility, antibacterial activity, anti-inflammatory, and antioxidant properties, ability to promote wound healing,

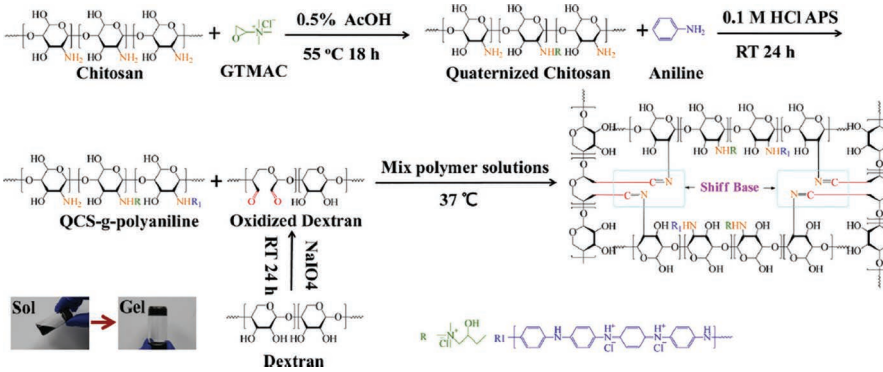


FIGURE 11.3 Schematic representation of the formation of QCS–polyaniline/oxidized dextran hydrogel. QCS represented quaternized chitosan and GTMAC is short for glycidyl trimethylammonium chloride.

mechanical properties, and a low frequency of wound dressing change should be designed. In this section, we discuss recent researches on CS-based biocomposites with multifunctional properties.

Zhao et al. prepared CS-based biocomposites composed of quaternized chitosan (QCS)-graft-polyaniline/oxidized dextran as shown in Figure 11.3 [57] to have antibacterial, conductive, and injectable hydrogels for joints skin wound healing because the electrical stimulation by polyaniline contributes to good cellular behaviors of electrical signal sensitive cells [58]. The results indicated that the hydrogels containing polyaniline showed higher antibacterial activity for *E. coli* and *S. aureus in vitro*, and showed enhanced antibacterial activity for *E. coli in vivo* with better cytocompatibility compared to the hydrogels without polyaniline due to the electroactive activity of the hydrogels, a suggestion of a new way to fabricate in situ forming antibacterial and electroactive hydrogels for skin tissue regeneration applications. They also prepared CS-based biocomposites by mixing QCS and benzaldehyde-terminated Pluronic F127 as shown in Figure 11.4 [59] to have antibacterial, injectable, rapid self-healing, extensibility, and compressibility hydrogels for joints skin wound dressing applications. The results indicated that the hydrogel dressings showed stretchable and compressive properties with good adhesive and fast self-healing ability to bear deformation. Also, the curcumin-loaded hydrogel showed antioxidant ability and accelerated wound healing with upregulation of vascular endothelial growth factor (VEGF) in a full-thickness mouse skin defect model, a suggestion of the possibility of wound dressing materials for joints skin wound healing. Furthermore, they prepared another CS-based biocomposite hydrogels based on QCS-graft-cyclodextrin (QCS-CD) and QCS-graft-adamantane (QCS-AD) as shown in Figure 11.5 [60] to have antibacterial, injectable self-healing, and photoconductive properties. The results indicated that the hydrogels had a conductivity value similar to that of the skin, rapid-healing property, and good antibacterial activity against *E. coli in vitro* and significantly accelerated the healing process of a full-thickness wound in *vivo*, indication of a promising wound dressing for full-thickness skin repair.

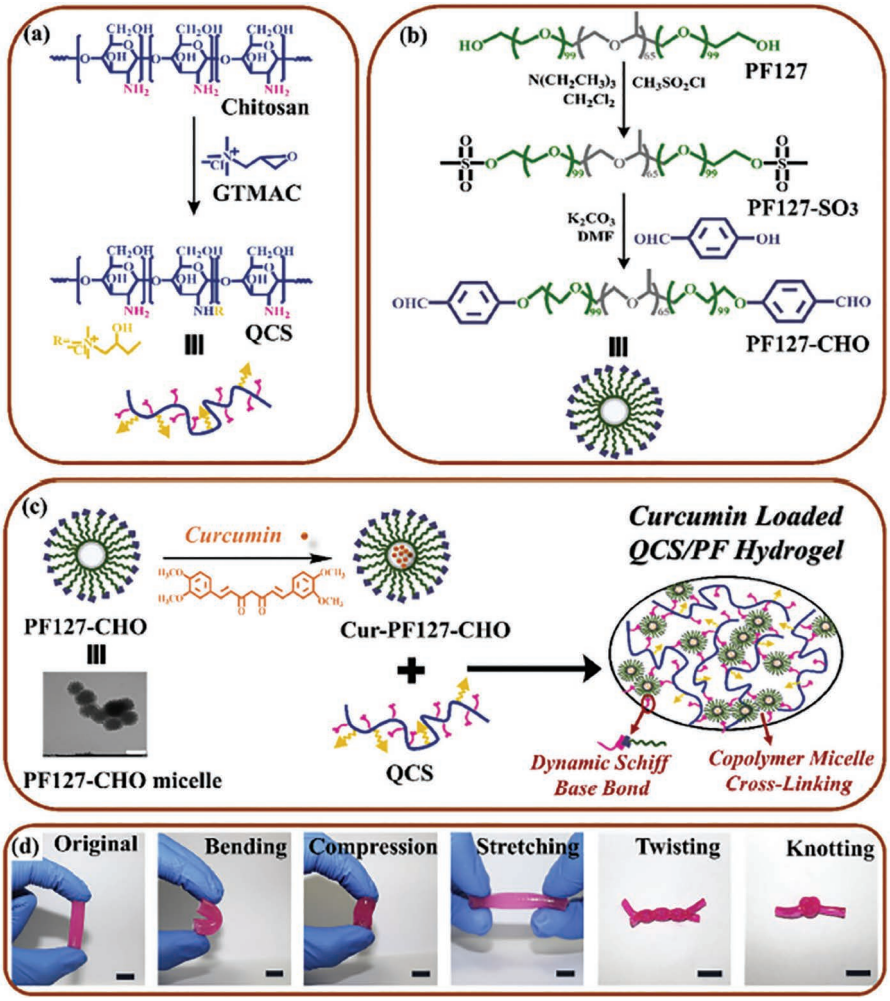


FIGURE 11.4 Schematic representation of Cur-QCS/PF hydrogel synthesis. (a) The synthesis scheme of QCS polymer. (b) PF127-CHO polymer. (c) Schematic illustration of Cur-QCS/PF hydrogel and TEM image of PF127-CHO micelles. Scale bar: 200 nm. (d) The original, bending, compression, stretching, twisting, and knotting shapes of rhodamine B dyed QCS/PF1.0 hydrogels. Scale bar: 1 cm.

Song et al. prepared CS-based biocomposite hydrogels containing cordycepin (CY) and CS after cross-linking by noncovalent bonds through a one-step freezing-thawing method for wound dressing application [61] because the CY as Chinese medicine has antibacterial, antioxidant, and suppression of inflammatory responsibility [61]. The results indicated that the hydrogels exhibited good biocompatibility, suitable water absorption, and remarkable antimicrobial effect with the desired mechanical strength *in vitro*. Also, the hydrogels showed a quicker re-epithelization of rat skin wounds, increased collagen deposition, and increased expression of epithelial regeneration

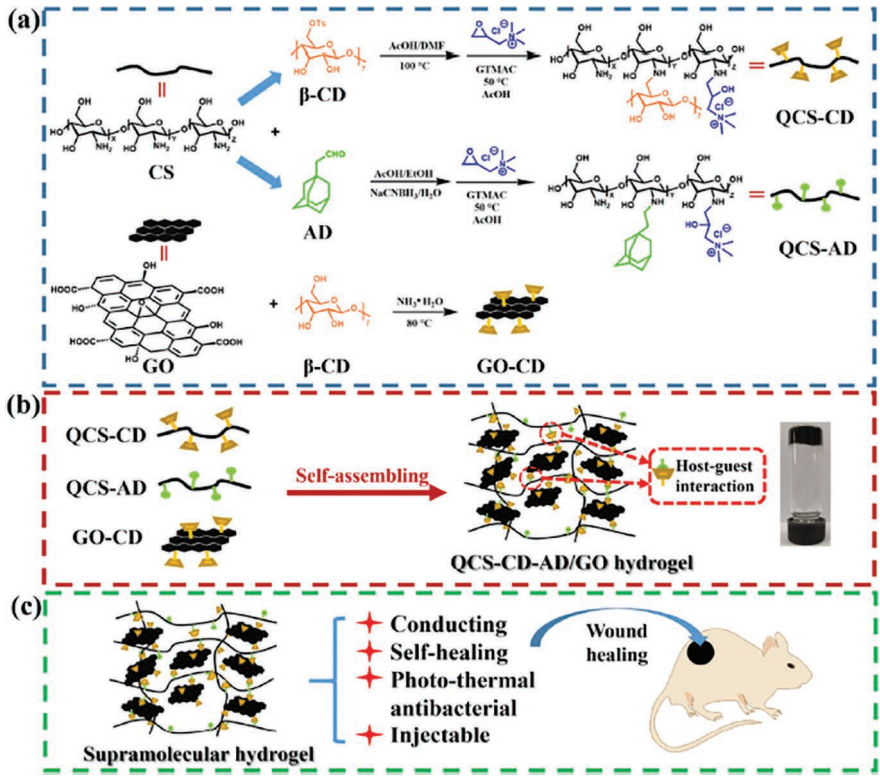


FIGURE 11.5 Schematic representation of QCS-CD-AD/GO supramolecular hydrogels preparation. (a) Preparation scheme of QCS-CD, QCS-AD, and GO-CD polymer. (b) QCS-CD-AD/GO supramolecular hydrogel. (c) Characteristic of QCS-CD-AD/GO hydrogel and the application in wound healing.

markers of laminin and involucrin in *vivo* compared with CS hydrogel due to the self-healing ability of the hydrogels.

Sundaram et al. prepared injectable CS-based biocomposites containing bioglass NPs and CS to control effective bleed when there is severe blood loss from major surgeries or skin wounds [62] because the bioglass can initiate the coagulation cascade [63]. The hydrogels showed injectable rapid blood clottable and cytocompatible properties in *vitro* and in *vivo*, an indication of potential hydrogel for getting effective bleeding control during critical situations.

11.4 CHITOSAN-BASED BIOCOMPOSITES FOR BONE TISSUE ENGINEERING

11.4.1 Requirement of Bone Tissue Engineering

Tissue engineering consisted of multidisciplinary science, including material engineering, molecular biology, and the clinical part to develop biological substitutes for

defective tissues or organs has recently become an important therapeutic strategy for the present and future medicine [1]. Among defected tissues, bone defect treatments are the most urgent problem in orthopedic surgery although autogenous and allogenous bone grafts, bone substitute material transplantation, and metal implants are currently performed [64]. However, several problems such as limited bone sources, potential infection and immune responses, biocompatibility, and mechanical properties limit their use in clinical practice. Therefore, bone tissue engineering will provide a new therapeutic method to solve the above-mentioned problems as an alternative.

In this section, we discuss the requirement of an ideal biomaterial scaffold among the indispensable components such as cells, growth factors, and scaffolds in bone tissue engineering. The biomaterials should have mechanical and appropriate properties, mimic natural bone structure to mineralize in *vivo*, function transporting and exchange to grow blood vessels, and be conductive to cell adhesion and to get normal proliferation and differentiation ability of the cells [65].

11.4.2 CS-based Biocomposite for Bone Tissue Engineering

In this section, we cover CS-based biocomposite to meet the requirement of bone tissue engineering mentioned in the previous section. Among the requirements, we focus on how to get the appropriate mechanical property, how to meet appropriate porosity, and how to function biological properties by the CS-based biocomposites for bone tissue engineering.

11.4.2.1 CS-Based Biocomposites Having Appropriate Mechanical Properties

Shokri et al. prepared CS-based biocomposites containing CS, bioactive glass (BG), and carbon nanotube (CNT) to overcome low mechanical strength and Young's modulus of CS itself for application of bone tissue engineering [66] because the presence of CNT can increase the compressive strength of the scaffolds. The results indicated that the compressive strength of CS was increased by adding CNT and was more increased with an increase of CNT in the scaffolds with an increase of attachment and proliferation of MG63 osteoblast cells on CS/BG/CNT scaffolds due to the surface formation of hydroxyapatite (HA) by the BG [67] although they did not perform *in vivo* study.

Nazeni et al. similarly prepared CS-based biocomposites containing CS, BG, and PLGA NPs to increase in mechanical strength of the scaffolds for bone tissue engineering application [68] because the PLGA as one of the synthetic polymers approved for clinical use due to the biocompatibility has relatively good processability [69]. The results indicated that the incorporation of the PLGA NPs increased the compression strength without affecting the morphologies of the scaffolds, a suggestion of a potential to be used as a controlled-release platform of related growth factor for bone tissue regeneration because the PLGA NPs have been used for sustained controlled drug release.

Pourhaghgouy et al. also prepared CS-based biocomposites containing CS and BG NPs by the freeze-casting method to increase the compressive strength and compressive modulus of the nanocomposite scaffolds for bone tissue engineering application [70].

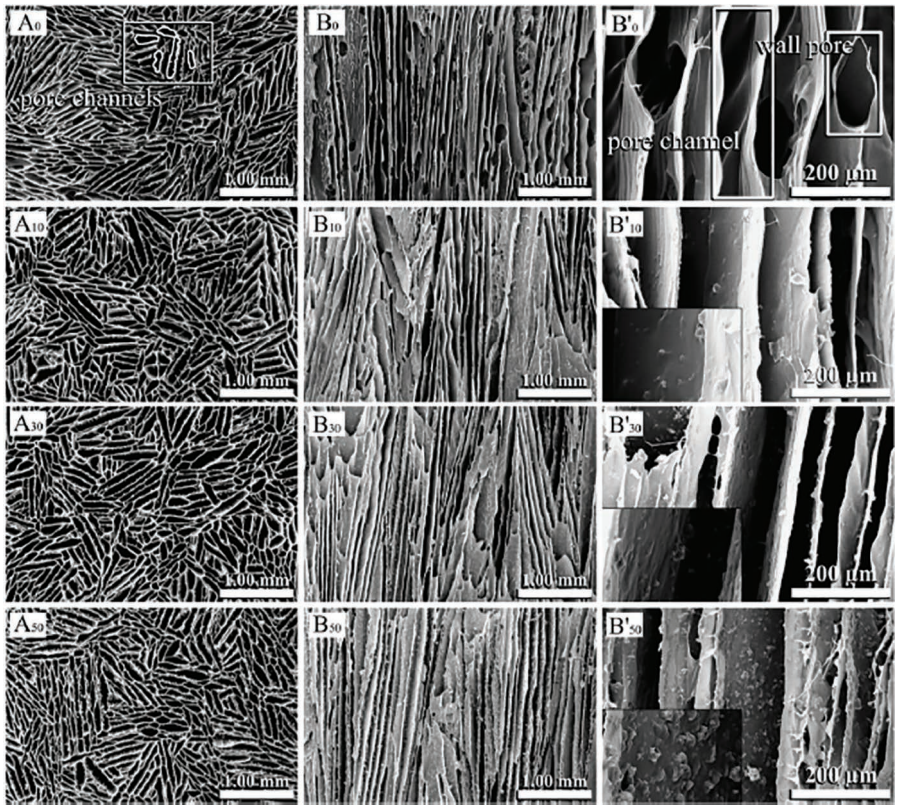


FIGURE 11.6 The SEM images taken from both (A) perpendicular and (B and B') parallel directions to the ice growth during the freeze-casting process. The images inserted in the B' series are the high magnification pictures of the B series which illustrate scaffolds' wall surfaces and the distribution of BGNPs on them. The subscripts indicate the BGNP contents of each scaffold (0, 10, 30, and 50 wt%).

The results indicated that the compressive strength and compressive modulus of the biocomposites increased 12 and 26 times, respectively, when 50 wt% of BG NPs were added into CS due to the unidirectional structure with a homogeneous distribution of BG NPs into CS scaffolds as shown in Figure 11.6 [70].

Zhang et al. prepared CS-based biocomposites containing CS, HA, and poly(3-hydroxybutyrate-co-3-hydroxy valerate) (PHBV) to enhance the mechanical property and biocompatibility of the biocomposite scaffolds [71] because the HA acts as a chelating agent for organizing the apatite-like mineralization [71] and the PHBV is a highly biocompatible polymer with high toughness [72]. The results indicated that the ultimate tensile strength of CS/PHBV/HA electrospun biocomposite nanofibers increased compared with that of CS/PHBV ones although the tensile strength of the CS/PHBV/HA depended on the content of HA and CS/PHBV/HA scaffold showed higher mineral deposition than that of PHBV one due to the synergistic effect of CS and HA, an indication of the potential to promote the regeneration of bone tissue.

Maji et al. also prepared CS-based biocomposites containing CS, GE, and HA to increase the mechanical strength of CS scaffold for bone scaffolds [73] because of having favorable mechanical properties by HA and having bioactivities by GE. The results indicated that the compressive strength of the biocomposites depended on the content of CS:GEL:HA in the composites and the highest compressive strength of the composites was obtained with a CS:GEL:HA weight ratio of 49-30-21 with having conductivity to mesenchymal stem cells adhesion due to the bioactive property of GEL, a suggestion of a successful contribution to the development of superior scaffolds for application of bone tissue engineering.

Kim et al. prepared CS-based biocomposites containing CS, AL, and HA NPs to increase the compressive strength and elastic modulus of CS/AL composite scaffolds for bone tissue engineering [74] because HA NPs can increase the mechanical properties due to homogenously dispersion of HA NPs in the biocomposites. The results indicated that the compressive strength and the elastic modulus of the biocomposites increased about 16 and about 20 times, respectively, compared with CS/AL composites with more differentiation and mineralization of the MC3T3-E1 cells when 70 wt% of HA NPs were added in the composite scaffolds. Similarly, Acevedo et al. added HA and titania NPs to increase the mechanical properties of CS-based biocomposites containing CS and GEL for application in bone regeneration [75] because both NPs increase thermophysical and mechanical properties of the composites. The results indicated that both NPs were homogeneously distributed in the CS/GEL composite membranes and Young's modulus of NPs-contained CS/GEL composite membranes increased by UV-irradiated cross-linking with the increase of the differentiation of MEF cells due to the osteoconductive property of both NPs although they did not check the mechanical properties of the composites without both NPs. Furthermore, Teimouri et al. added zirconia (ZrO_2) NPs to increase enhanced mechanical properties of CS-based biocomposites containing CS and silk fibroin (SF) [76] because ZnO_2 NPs have remarkable mechanical properties [77]. The results indicated that the compressive strength of CS/SF scaffolds increased with an addition of ZnO_2 without cytotoxicity although they used human gingival fibroblast cells *in vitro* cytotoxicity and cell attachment for dental tissue engineering.

HA-contained biocomposites have been used for their application in bone tissue engineering because of the HA-induced osteoconductivity [78]. However, beta-tricalcium phosphate (β-TCP) as an alternative ceramic has been used because the β-TCP has a ten times higher degradation rate than HA [78] with the promotion of osteogenesis and improvement of bone regeneration [79].

Serra et al. prepared and characterized CS-based biocomposites containing CS, GEL, and β-TCP to get the osteogenesis of CS/GEL composite scaffolds for bone tissue engineering due to the fast dissolution and absorption of the β-TCP [80] although low mechanical properties of the biocomposites can be overcome by ionic crosslinking with sodium tripolyphosphate. The results indicated that the incorporation of GEL and/or β-TCP in the CS scaffolds increased their compressive strength by about 70% and enhanced mineral deposition on the biocomposite scaffolds immersed in standard simulated body fluid (SBF) solution with antimicrobial activity against *S.* aureus, a suggestion of production of biomimetic scaffolds to improve bone regeneration. Similarly, Puvaneswary et al. prepared CS-based biocomposites containing

CS, fucoidan (FU), and β-TCP to increase proliferation and mineralization in human bone marrow stromal cells for bone tissue engineering [81] because the FU showed mineralization in human adipose-derived stem cells (hADSs) [82]. The results indicated that CS/FU/β-TCP biocomposite scaffolds showed higher compressive strength and modulus than those of CS/β-TCP scaffolds due to the addition of FU and increased osteogenic differentiation of hMSCs due to the increase of released osteocalcin by the FU.

11.4.2.2 CS-Based Biocomposites Having Appropriate Porosity

The CS-based biocomposites for bone tissue engineering application should have the ability to develop a cell-based repairing biomaterial for the regeneration of bone defect [83] because the main function of the scaffolds is to facilitate the making of bone tissues of preferred size, shape, and function by serving as a structural template [84] with having adequate porosity to get cell adhesion, proliferation, and nutrient transfer. Several approaches such as gas foaming, freeze-drying, particle leaching, thermally induced phase separation, electrospinning, and three-dimensional (3D) printing have been tried to fabricate the scaffolds having the appropriate porosity. In this section, we want to discuss the characteristics of each method.

11.4.2.2.1 Gas Foaming

The gas foaming method was used to avoid organic solvents by using inert gas foaming agents such as carbon dioxide and nitrogen to pressurize molded polymers with water until they are saturated and full of gas bubbles [85]. This method generally makes sponge-like structures with pore sizes of 30~700 μm and porosity of up to 85% [86]. The disadvantages of this method have the use of excessive heat during compression molding, non-interconnected pore structures, and nonporous skin layers at the scaffold surface [85].

Gravel et al. prepared macroporous CS-based biocomposites containing CS and coral [87] by gas foaming because the coral mainly composed of calcium carbonate can produce carbon dioxide by the reaction between the coral and acidic CS solvent. The results indicated that the average pore sizes of the CS/coral scaffolds were from 80 to 400 μm according to the weight of coral from 0 to 75 wt% whereas the porosity decreased from 91% to 75 wt% with an increase of compressive modulus and fast MSCs adhesion due to the remained coral particles in the scaffolds. They also investigated responses of MSCs to the CS/coral biocomposites prepared by the gas-forming agent to check their scaffolding potential *in vitro* bone regeneration [88]. The results indicated that the CS/coral biocomposite scaffolds with a high content of coral showed higher cell number, alkaline phosphatase (ALP) activity, and osteocalcin (OC) protein expression compared to CS itself.

11.4.2.2.2 Freeze-Drying

The freeze-drying method known as lyophilization can be applied by several processes such as the dissolving of used scaffolds in a suitable solvent, cooling down of solved scaffolds below their freezing point for leading to the solidification of the solvent, and finally evaporation of the solvent via sublimation for making dry scaffolds [85]. The advantages of this method are to avoid high temperatures that can affect

the activity of the incorporated growth factors and easily control pore size by tuning the freezing regime. On the other hand, the disadvantages of this method are lengthy time scales, the use of cytotoxic organic solvents, high energy consumption, and the production of small and irregular pore sizes [89].

Kalanthai et al. prepared CS-based biocomposite scaffolds containing graphene oxide (GO), CS, COL, and cross-linked AL by the freeze-drying method for bone tissue engineering [90] because the addition of GO in the crosslinked AL/CS/COL scaffolds increased the mechanical strength and improved osteogenic differentiation *in vivo* [91]. The results indicated that the GO/CS/COL/AL scaffolds exhibited interconnected pores of 0~250 μm range and a significant MC3T3 cell attachment compared to CS/COL/AL ones although they did not show any effect on the osteogenic ability of osteoblasts.

Demir et al. prepared CS-based biocomposite scaffolds containing CS, montmorillonite (MMT), and strontium (ST) by the freeze-drying method as bone tissue engineering scaffold [92] because the MMT has a good cation-exchange ability [93] and the ST stimulated pro-osteoblast proliferation and activity [94]. The results indicated that the CS/MMT/ST scaffolds showed highly porous morphologies with interconnected pores and displayed significantly higher DNA concentrations of the human osteoblast cells due to the incorporation of ST in the scaffolds.

Pineda-Castillo et al. prepared and characterized CS-based biocomposite scaffolds containing CS, PVA, and HA by the freeze-drying method for bone tissue regeneration [95] because the HA improved osteoconductivity [96]. The results indicated that the CS/PVA/HA scaffolds showed uniform pore sizes of 142~519 μm range that had been described as optimal bone defect regeneration without cytotoxicity due to pore formation with interconnected pores by the presence of CS in the scaffolds.

Peng et al. prepared CS-based biocomposite scaffolds containing CS, mesoporous calcium silicate (MCS), and lanthanum (LA) via the freeze-drying method for bone tissue engineering [97] because the MCS accelerated *in vivo* bone tissue regeneration [98] and the LA ions enhanced the proliferation and osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells (BMSCs). The results indicated that the CS/MCS/LA scaffolds showed 3D macropores with a size of around 200 μm and significantly induced the osteogenic differentiation of BMSCs *in vitro* and promoted new bone deposition *in vivo* rat cranial bone defect models, a suggestion of application potential for the bone defect.

Shi et al. prepared CS-based CS biocomposites containing CS, dopamine-modified AL (DA-AL), and HA NPs by integrative layering method with further crosslinking by Ca^{2+} ions for bone tissue engineering [99] because the as-prepared CS/DA-AL/HA NPs scaffolds make gradient scaffolds for appropriate degradation rate to get fast bone regeneration. The results indicated that the CS/DA-AL/HA NPs scaffolds had integrated layer structures and high porosity at around 77.5% and showed good adhesion of chondrocytes and fibroblasts *in vitro*, and promoted the regeneration of the bone tissue with the acceleration of the repair of the bone defects in white New Zealand rabbits.

Recently, Sadeghinia et al. prepared CS-based biocomposites containing CS, GEL, clinoptilolite (CLN), and HA NPs by the freeze-drying method for bone tissue engineering [100] because the CLN showed an immunostimulatory effect with an increase

of mechanical strength [101]. The results indicated that the CS/GEL/CLN/HA NPs scaffolds showed highly porous morphologies with pore sizes of $200 \pm 100\,\mu m$ and showed increased biomineralization and enhanced mechanical strength without cytotoxicity due to the presence of CLN and HA NPs in the scaffolds.

11.4.2.2.3 Particle Leaching

The particle leaching method is firstly to dissolve the scaffolds by the solvent with uniformly distributed salt particles, to evaporate the solvent with leaving the salt particles-loaded scaffolds, and finally to immerse in water for leaching out to make a porous structure [85]. The advantages of this method are relatively easy to make pores with the sustainable equipment cost and to make high scaffold porosity with the feasibility for tuning pore size [85]. On the other hand, the disadvantage of this method is only to form simple shape scaffolds and harmful to cells by the remained solvent [102].

Jamalpoor et al. prepared CS-based biocomposite scaffolds containing CS, GEL, and HA NPs by the particle leaching method using NaCl as porogen for bone tissue engineering [103] to make 3D scaffolds with optimum porosity and pore size with the bone matching mechanical strength. The results indicated that the CS/GEL/HA NPs scaffolds showed highly interconnected porous structures with a mean pore size of 140–190 μm and increased GEL content in the scaffolds improved attachment, infiltration, and proliferation of Saos 2 cells.

Ruixin et al. prepared CS-based biocomposite scaffolds containing CS and HA microparticles by the particle leaching method using spherical paraffin as porogen for bone regeneration because the HA can improve the bioactivity and bone-bonding ability. The results indicated that the pore of the CS/HA microparticle scaffolds showed interconnected spherical macropores with the increase of pore by the increase of porogen although there were some pores deformed due to the deformation of the paraffin by the stirring. Also, they prepared CS/HA NPs or CS/HA microparticle scaffolds using the same porogen to compare biocompatibility between both scaffolds [104]. The results indicated that both scaffolds showed interconnected spherical pores without differences in structural parameters and good biocompatibility in MC3T3-E1 cells without significant difference of cell viabilities between both scaffolds.

Wang et al. prepared hydroxyethyl CS (HCS)-based biocomposite scaffolds containing HCS and chemical crosslinked CEL by the particle leaching method using silicon dioxide particles as porogen for bone tissue engineering [105] because the crosslinked CEL can enhance the compression modulus and elasticity. The results indicated that HCS/crosslinked CEL scaffolds showed bubble-like macropore structure with a pore size of 100~250 μm by the removal of porogen SiO_2 particles and micropore structure with a pore size of several tens of microns by the sublimation of ice crystals formed during freeze-drying and facilitated the attachment, spreading, and osteoblastic MC3T3-E1 cells due to the addition of HCS in the scaffolds, suggestion of promising scaffolds for bone tissue engineering application.

11.4.2.2.4 Thermally Induced Phase Separation

The TIPS is one of the low-temperature processes because a scaffold solution is quenched and undergoes a liquid/liquid phase separation: one scaffold-rich and the other scaffold-poor [85]. The scaffold-rich phase solidifies whereas the scaffold poor

one is removed, leaving a highly porous and nanoscale fibrous network [85]. The advantage of this method is a low-temperature process that favors the incorporation of growth factors in the nanoscale structure for serving as a template of drug-loaded NPs in the scaffolds. On the other hand, the disadvantage of this method is to combine with another method for making macroporous structures in the scaffolds.

Zhang et al. prepared biomimetic osteochondral scaffolds containing the oriented cartilage layer designed to mimic native cartilage tissue and fabricated with cartilage matrix-CS using TIPS, a compact layer designed to mimic the calcified-layer structure of natural cartilage and 3D-printed core-sheath structured-bone layer fabricated with PLGA/β-TCP-COL by low-temperature deposition method [106]. The results indicated that the three part-combined scaffolds exhibited good mechanical properties with hydrophilicity and BMSC-loaded scaffolds regenerated trabecular bone formed in the subchondral bone defect model of goat, a suggestion of possibility for future clinical application in bone-defect repair.

Rahman et al. prepared crosslinked-CS-based biocomposite scaffolds containing CS, COL 1, and HA by the TIPS method for restoration of defected maxillofacial mandible bone [107]. The results indicated that the scaffolds exhibited irregular porous structures with moderate interconnected structures with a pore diameter of 111.8~212.6 μm for CS/COL 1/HA although the pore diameters of the scaffolds were decreased after cross-linking and de-hydrothermal cross-linked CS/COL 1/HA scaffolds showed the restoration of defected bone in the rabbit.

Recently, Erickson et al. prepared CS-based biocomposite bilayer scaffolds consisted of CS-HYA (hyaluronic acid) cartilage layer and CS-AL HA bone layer by the TIPS method for osteochondral tissue regeneration [108]. The results indicated that the scaffolds showed an open pore network with interconnected structures although the pore diameter of the scaffolds depended on the content of used CS, AL, and HA, and gene expression related with osteogenesis and chondrogenesis increased after co-culture with chondrocyte-like SW-1353 and osteoblast-like MG 63 in the scaffolds.

11.4.2.2.5 3D Printing

The 3D printing method has been used to be a rational strategy to make 3D scaffolds for overcoming the limitations of traditional methods because direct or indirect 3D printing methods can provide precise control over pore interconnectivity, size, internal architecture, and external shape of the 3D scaffolds, and can create the 3D structure with the size of the defective part and the correct anatomical shape [109]. The advantages of this 3D printing method enable not only mass customization of goods on a large scale but also smaller production runs with a high degree of customization [110]. On the other hand, the disadvantages of this 3D printing method are how to get reproducibility of the various scales and complexities of engineered tissues.

Demirtas et al. prepared CS-based biocomposite hydrogel scaffolds containing CS and HA NPs by the extruder-based bioprinter for bone tissue engineering because 3D patterning of cells and growth factors as a bioprintable form can fabricate living tissue and organs for tissue engineering [111]. The results indicated that bioprinted-hydrogels MC3T3-E1 pre-osteoblast cell-laden CS/HA NPs showed peak expression levels for early and late stages osteogenic markers with high cell viability

and the loaded cells in the CS/HA NPs hydrogels had higher cell proliferation and differentiation compared with AL itself, a suggestion of applicability and printability of CS/HA NPs hydrogel as a bioprinting solution.

Dong et al. prepared CS-based biocomposite hydrogel scaffolds containing CS and PCL by 3D printing to improve the cell seeding efficiency and osteoinductivity in the PCL scaffolds because an injectable thermo-sensitive CS hydrogel can be incorporated into 3D-printed PCL scaffold for bone tissue engineering application [112]. The results indicated that greater retention and proliferation in rabbit BMSCs and bone morphogenetic protein-2 (BMP-2)-laden CS/PCL hydrogel scaffolds were obtained than PCL itself and stronger osteogenesis with the bone-matrix formation was shown in the CS/PCL hybrid system than PCL one *in vitro* after 2-week, a suggestion of a promising platform for bone tissue engineering due to their ability to load cells and drugs, and excellent mechanical strength.

Yang et al. prepared CS-based biocomposite scaffolds containing quaternized CS (QCS)-grafted PLGA and HA by the 3D printing method to inhibit bacterial infection and to promote bone regeneration [111] because the infection is the pivotal cause of nonunion in the bone defect. The results indicated that QCS-grafted PLGA/HA scaffolds significantly exhibited improved antimicrobial and osteoconductive properties *in vitro*, and enhanced anti-infection and bone regeneration abilities in infected bone defect rats or rabbit models, a suggestion of a promising dual-functional scaffold for repairing bone defect under infection.

Tsai et al. prepared CS-based biocomposite scaffolds containing CS, titanium alloy (TA), and magnesium-calcium silicate (MCS) by the 3D printing method for orthopedic application [113] because the bioactivity of the TA/MCS can be improved using the simple immersion method by the CS. The results indicated that CS/TA/MCS scaffolds exhibited enhanced cell adhesion, proliferation, and differentiation *in vitro*, and enhanced bone regeneration and in growth at the critical size bone defects in the rabbit model, an indication of induction of micro-environment for bone regeneration by the simple immersion method.

Chen et al. prepared CS-based biocomposite scaffolds containing CS, GEL, and Mg (Mg)-substituted HA (Mg-HA) prepared by biomimetic mineralization of COL 1 and citric acid as the bi-template via 3D printing method for bone regeneration [114] because the substitution of Mg for cations reduce the crystallinity of HA without affecting the size and structure of HA. The results indicated that CS/GEL/Mg-HA scaffolds exhibited higher cell attachment, proliferation rate, increased expression of ALP activity, and osteogenic related genes such as osteocalcin, runt-related transcription factor 2, and COL 1, as an indication of a potential candidate of biocomposite scaffolds in bone tissue engineering.

Recently, Chen et al. prepared CS-based biocomposite scaffolds containing carboxymethyl chitosan (CMCS), HA, and polydopamine (PDA) by the 3D printing method [115] for repairing bone defects because the PDA enhanced cell adhesion [116] with high biocompatibility and improved stability of the bound materials in the surfaces. The results indicated that the CMCS/HA/PDA scaffolds exhibited a porous structure with the size of $415 \pm 87\,\mu m$ and $69.5\% \pm 4.6\%$ porosity and effectively stimulated new bone formation within the femoral lacuna defect site of rabbits after 12 weeks, a suggestion of a remarkable potential new scaffold for repair of bone defects.

11.4.2.2.6 Electrospinning

The electrospinning method is to use electrical charges for drawing fine fibers up to the nanometer scale and creating a nanofibrous architecture [85]. Generally, there are four major components such as a spinner with a metallic needle, a high-voltage power supply, a syringe pump, and a grounded collector.

Electrospinning has been used to fabricate scaffolds with both micro and nanostructures for tissue engineering application although the electrospun scaffolds have weak mechanical properties due to the high porosity and nonaligned microfibers.

Jing et al. at first prepared parallel-aligned poly(propylene carbonate) (PPC) microfibers by electrospinning, treated oxygen plasma, and introduced CS nanofibers to increase the mechanical properties of the composite scaffolds for the application of tissue engineering [117]. The results indicated that Young's modulus of the PPC increased by about 26% after the treatment of the CS.

Nanofibers under dry conditions with a superior cell response were obtained whereas the difference between PPC and PPC/CS scaffolds was not much obtained under wet conditions although they used 3T3 cells instead of bone cells.

11.4.3 CS-Based Biocomposites Having Appropriate Biological Functions

Growth factors have been widely used for bone tissue engineering because they are responsible for cellular behaviors such as proliferation, migration, and differentiation. Therefore, various growth factors have been loaded in CS-based biocomposite scaffolds after physically or chemically loaded of growth factors into the scaffolds [118]. In this section, we discuss the biological functions of bone-related cells after loading growth factors into the scaffolds. Among the growth factors, BMP-2 has been extensively used in bone tissue engineering because it is approved by the USA FDA for bone graft fusion due to the safety and efficient bone growth better than any BMPs [119].

Sobhani et al. prepared calcium phosphate/polyphosphazene scaffolds containing BMP-2-loaded CS microspheres in bone tissue engineering [120] because loaded BMP-2 into the CS microspheres can be sustainably released to induce an osteoblast proliferation. The results indicated that BMP-2-loaded scaffolds increased the osteogenic differentiation ability of BMSCs compared with the scaffolds alone. Similarly, Bastami et al. prepared GEL/β-TCP/COL scaffolds containing BMP-2-loaded CS NPs for bone tissue engineering [121] because loaded BMP-2 into the CS NPs can be sustainably released to get differentiation of human buccal fat pad-derived stem cells (hBGPSCs). The results indicated that the BMP-2-loaded scaffolds showed an enhanced osteoinductive graft compared with the scaffolds alone due to the sustained delivery of BMP-2 in a therapeutic window. Also, Deng et al. prepared PLGA/HA NPs scaffolds containing BMP-2-loaded CS NPs for bone tissue engineering [122] because the sustained release of BMP-2 from the CS NPs induced bone regeneration due to the osteogenic effect of the BMP-2. The results indicated that the BMP-2-loaded scaffolds showed faster new bone formation in a rabbit mandible bone defect model without any significant inflammatory response compared with scaffolds alone due to the osteogenesis effect by the released BMP-2.

Tong et al. prepared transforming growth factors-β1(TGF-β1)-loaded CS/SF 3D scaffolds for bone tissue engineering [123] because the TGF-β1 induces the differentiation and proliferation of osteoblasts and BMSCs [124]. The results indicated that the TGF-β1-loaded scaffolds significantly more enhanced the growth and proliferation of BMSC in a tissue-dependent manner *in vitro*, and exhibited extensive osteoconductivity with the host bone, and enhanced new bone formation after implant in rabbit mandibles model after 8 weeks compared with the scaffolds alone due to the effect of released TGF-β1 from the scaffolds, a suggestion of a promising potential to be applied in orthopedic surgery.

Oryan et al. prepared platelet gel (PG)-loaded CS/GEL biocomposite scaffolds to regenerate bone defect [125] because the PG contains angiogenic, mitogenic, and osteogenic growth factors in their α-granules [126]. The results indicated that the PG-loaded CS/GEL scaffolds showed significantly higher new bone formation, bone volume, the density of osseous and cartilaginous tissue, and numbers of osteons in critical-sized radial bone defect of a rat after 8 weeks compared with the CS/GEL scaffolds alone due to the regenerative effect by the incorporated PG in the scaffolds. Similarly, Liao et al. prepared PRP-loaded thermo-gelling hydrogel, HYA-g-CS-g-poly (*N*-isopropyl acrylamide) (HYA-CS-PNIP) after embedding of biphasic calcium phosphate (BCP) and rabbit adipose-derived stem cells (rASCs) to get osteoinductive properties [127]. The results indicated that the PRP- and rASCs-loaded injectable thermo-sensitive HYA-CS-PNIP/BCP scaffolds showed increased cell proliferation, alkaline phosphatase activity, increased calcium deposition, and upregulated expression of osteogenesis *in vitro*, and induced more new bone formation at the rabbit critical size calvarial bone defect model, a suggestion of a promising biocomposite hydrogel scaffolds for bone tissue engineering.

In some cases, combined growth factors can be used to get synergistic or additive effects of growth factors for promotion of the bone regeneration. Wang et al. prepared stromal cell-derived factor (SDF-1)- and BMP-2-loaded CS/agarose (AG)/GEL scaffolds synthesized via gelation method using cross-linked CS, AG, and GEL, after modified by CS/HEP NPs [128] because the SDF-1 plays a critical role in the mobilization of MSCs and the BMP-2 plays a critical role in osteogenesis of MSCs. The results indicated that both growth factors-loaded and CS/HEP NPs-modified scaffolds retained migration activity of MSCs and strongly induced differentiation towards osteoblasts *in vitro*, and showed a continuous chemotactic response of MSCs in nude mice after subcutaneous implantation of two growth factors-loaded and CS/HEP NPs-modified scaffolds into the back of the mouse, a suggestion of attractive scaffolds to promote bone repair and regeneration. Similarly, Dou et al. prepared COL/HA scaffolds containing VEGF- and BMP-2-loaded CMCS microspheres for bone tissue engineering [129] because the VEGF promotes vascular regeneration and improves the activity of osteoblasts, and the BMP-2 promotes bone regeneration [130]. The results indicated that both growth factors-loaded scaffolds showed more conductivity to the differentiation of pre-osteoblasts *in vitro* and promoted the formation of blood vessels and the formation of COL *in vivo* due to the sequential release of the double growth factors. Furthermore, Sadeghinia et al. prepared CS/GEL/HA NPs biocomposite scaffolds combined with PRP and fibrin glue (FG) to enhance proliferation and differentiation of seeded human dental pulp stem cells

(HDPSCs) for dental bone tissue engineering [100] because the PRP enhanced osteogenesis and bone formation [131], and the FG showed increase osteoconductivity and biocompatibility [132]. The results indicated that both growth factors-loaded scaffolds improved adhesion formation of bone minerals, and BMP-2 gene expression of seeded HDPSCs compared with the scaffolds alone.

11.5 CONCLUSION, OPPORTUNITY, AND CHALLENGE

The CS and CS derivatives have been used in various biomedical applications such as wound dressing, tissue engineering, and drug delivery carriers due to the unique biological properties, extensive adjustment of physicochemical properties, and easy processability although they have relatively poor mechanical, thermal, not-enough biological and barrier properties. In this regard, CS-based biocomposites after the addition of two or more biomaterials should be desirable.

Considering the acceptable mechanical property, prevention of bacterial invasion, absorption of excess exudates, adequate water and oxygen exchange, and the existence of anti-inflammation and antioxidants for wound dressing application, no single wound dressing can meet all requirements, thus the challenge is to develop novel CS-based wound dressings that can positively affect all or most wound types. The current challenge is how to develop multi-functional wound dressings for simultaneously providing therapeutic properties such as adhesion, absorption, mechanical strength, antibacterial property, and a moist wound environment using CS-based biocomposites because the variation in the rate of production of wound exudates and the variation in the appearance of the wound surface should be considered, although the advancement of tissue engineering technologies can help limitation of the single wound dressing. Also, the emergence of novel CS-based biocomposites to mimic the skin environment, conditions and structure is very important in the management of various wound types. Furthermore, drug- and growth factors-loaded CS-based biocomposites can stimulate wound healing responses to promote optimal treatment.

Tissue engineering technique to mimic the ECM for the regulation of cellular behaviors is generally satisfied with 3D constructs as well as more closely to mimic the *in vivo* micro/nanoarchitecture for improving the function of tissue-engineered constructs because highly porous and fortified 3D molds are very critical for the bone regeneration. Biodegradability of the 3D scaffolds is very important in harmony with bone regeneration although the choice of appropriate biomaterials depends on the particular site of application. Multifunctional injectable scaffolds including micro-/nano-hydrogels to deliver cells and growth factors for minimal surgical intervention are very promising because traditional tissue-engineered scaffolds provide painful procedures to create lesions and longer healing times. In this regard, material scientists to design scaffold architecture, polymer chemists to get optimum physicochemical properties, cell biologists for the regulation of cellular behaviors, and clinicians for the successful implant in clinical trials should be harmonized.

This review covers an overview of the current status of CS-based biocomposites in wound dressing and bone tissue engineering applications. It also discussed their current challenges and opportunities for future researches. We expect that this

review will be truly helpful for the researchers working in the field of CS-based biocomposite-related approaches.

REFERENCES

1. Kim I.Y., Seo S.J., Moon H.S., Yoo M.K., Park I.Y., Kim B.C., Cho C.S. Chitosan and its derivatives for tissue engineering applications, *Biotechnology Advances* 2008;26:1–21.
2. Krajewska B. Membrane-based processes performed with use of chitin/chitosan materials. *Separation and Purification Technology* 2005;41:305–312.
3. Eugene K., Lee Y.L. Implantable applications of chitin and chitosan, *Biomaterials* 2003;24:2339–2349.
4. Domish M., Kaplan D., Skaugrud O. Standards and guidelines for biopolymers in tissue-engineered medical products: ASTM alginate and chitosan standard guides. *Annals of the New York Academy of Sciences* 2001;944:388.
5. Muzzarelli R.A.A. Chitins and chitosans for the repair of woundedskin, nerve, cartilage and bone. *Carbohydrate Polymers* 2009;76:167–182.
6. Sudheesh Kumar P.T., Abhilash S., Manzoor K., Nair S.V., Tamura H., Jayakumar R. Preparation and characterization of novel-chitin/nano silver composite scaffolds for wound dressing applications. *Carbohydrate Polymers* 2010;80:761–777.
7. Vacanti C.A. The history of tissue engineering. *Journal of Cellular and Molecular Medicine* 2006;10:569–576.
8. Krajewska B. Membrane-based processes performed with use of chitin/chitosan materials. *Separation and Purification Technology* 2005;41:305–312.
9. Dev A, Binulal NS, Anitha A, Nair SV, Furuike T, Tamura H, Jayakumar R. Preparation of novel poly(lactic acid)/chitosan nanoparti-cles for anti HIV drug delivery applications. *Carbohydrate Polymers* 2010;80:833–838.
10. Yi H., Wu L.Q., Bentley W.E., Ghodssi R., Rubloff G.W., Culver J.N., Payne G.F. Biofabrication with chitosan. *Biomacromolecules* 2005;6:2881–2894.
11. Muramatsu K., Masuda S., Yoshihara S., Fujisawa A. In vitro degradation behavior of freeze-dried carboxymethyl-chitin sponges processed by vacuum-heating and gamma irradiation. *Polymer Degradation and Stability* 2003;81:327–332.
12. Drury J.L., Mooney D.J. Hydrogels for tissue engineering: Scaffold design variables and applications. *Biomaterials* 2003;24:4337–4351.
13. Prabaharan M., Mano J.F. Chitosan-based particles as controlled drugdelivery systems. *Drug Delivery* 2005;12:41–57.
14. Anitha A., Divya R.V.V., Krishna R., Sreeja V., Selvamurugan N., Nair S.V., Tamura H., Jayakumar R. Synthesis, characterization, cyto-toxicity and antibacterial studies of chitosan, O-carboxymethyl, N, O-carboxymethyl chitosan nanoparticles. *Carbohydrate Polymers* 2009;78:672–677.
15. Jayakumar R., Prabaharan M., Nair S.V., Tamura H. Novel chitin and chitosan nanofibers in biomedical applications. *Biotechnology Advances* 2010;28:142–150.
16. Jayakumar R., Reis R.L., Mano J.F. Synthesis and characterization of pH-sensitive thiol-containing chitosan beads for controlled drug delivery applications. *Drug Delivery* 2007;14:9–17.
17. Ehrlich H., Krajewska B., Hanke T., Born R., Heinemann S., Knieb C., Worch H. Chitosan membrane as a template for hydroxyap-atite crystal growth in a model dual membrane diffusion system. *Journal of Membrane Science* 2006;273:124–128.
18. Khalil H.P.S.A., Saurabh C.K., Adnan A.S., Fazita M.R.N., Syakir M.I., Davoudpour Y., Rafatullah M., Abdullah C.K., Haafiz M.K.M., Dungani R. A review on chitosan-cellulose blends and nanocellulose reinforced chitosan biocomposites: Properties and their applications. *Carbohydrate Polymers* 2016;150:216–226.

19. Suarato G., Rosalia Bertorelli R., Athanassiou A. Borrowing From Nature: Biopolymers and Biocomposites as Smart Wound Care Materials. *Frontiers in Bioengineering and Biotechnology* 2018;6:137. doi:10.3389/fbioe.2018.00137W-6.
20. Parsons D., Bowler P.G., Myles V., Jones S. Silver antimicrobial dressings in wound management, a comparison of anti-bacterial physical and chemical characteristics. *Wounds* 2005;17:222–232.
21. Mayet N., Choonara Y.E., Kumar P., Tomar L.K., Tyagi C., Du Toit L.C., Pillay V. A comprehensive review of advanced biopolymeric wound healing systems. *Journal of Pharmaceutical Sciences* 2014;103:2211–2230. doi:10.1002/jps.24068.
22. Kallaiinen L.K., Gordillo G.M., Schlanger R.K., Sen C. Topical oxygen as adjunct to wound healing, a clinical case series. *Pathophysiology* 2003;9:208–216.
23. Lloyd A.W. Interfacial bioengineering to enhance surface biocompatibility. *Medical Device Technologies* 2002;13:18–21.
24. Boateng J.S., Matthews K.H., Stevens N.E., Eccleston G.M. Wound healing dressings and drug delivery systems: A review. *Journal of Pharmaceutical Sciences* 2008;97: 2892–2923.
25. Harkins A.L., Duri S., Kloth L.C., Tran, C.D. Chitosan-cellulose composite for wound dressing material. Part 2. Antimicrobial activity, blood absorption ability, and biocompatibility. *Journal of Biomedical Materials Research Part B* 2014;102(6):1199–1206.
26. Leuner C., Dressman J. Improving drug solubility for oral delivery using solid dispersions. *European Journal of Pharmaceutics and Biopharmaceutics* 2000;50(1):47–60.
27. Jiang Q., Zhou W., Wang J., Tang R., Zhang D., Wang X. Hypromellose succinate-crosslinked chitosan hydrogel films for potential wound dressing. *International Journal of Biological Macromolecules* 2016;91:85–91.
28. Kim I.Y., Yoo M.K., Kim B.C., Kim S.K., Lee H.C., Cho C.S. Preparation of semi-interpenetrating polymer networks composed of chitosan and poloxamer. *International Journal of Biological Macromolecules* 2006;38:51–58.
29. Kim I.Y., Yoo M.K., Seo J.H., Park S.S., Na H.S., Lee H.C., Kim S.K., Cho C.S. Evaluation of semi-interpenetrating polymer networks composed of chitosan and poloxamer for wound dressing application. *International Journal of Pharmaceutics* 2007;341:35–43.
30. Akhavan-Kharazian N., Izadi-Vasafi H. Preparation and characterization of chitosan/gelatin/nanocrystalline cellulose/calcium peroxide films for potential wound dressing applications. *International Journal of Biological Macromolecules* 2019;133:881–891.
31. Gao Y., Zhang X., Jin X. Preparation and properties of minocycline-loaded carboxymethyl chitosan gel/alginate nonwovens composite wound dressings. *Marine Drugs* 2019;17(10):575. doi:10.3390/md17100575.
32. Pathalamuthu P., Siddharthan A., Giridev V.R., Victoria V., Thangam R., Sivasubramanian S., Savariar V., Hemamalini T. Enhanced performance of Aloe vera incorporated chitosan-polyethylene oxide electrospun wound scaffold produced using novel Spirograph based collector assembly. *International Journal of Biological Macromolecules* 2019;140:808–824.
33. Rahmani H., Najafi S.H.M., Ashori A., Fashapoyeh M.A., Mohseni F.A., Torkaman S. Preparation of chitosan-based composites with urethane cross linkage and evaluation of their properties for using as wound healing dressing. *Carbohydrate Polymers* 2020;230:115606. doi:10.1016/j.carbpol.2019.115606.
34. Khalili R., Zarrintaj P., Jafari S.H., Vahabi H., Saeb M.R. Electroactive poly (p-phenylene sulfide)/r-graphene oxide/chitosan as a novel potential candidate for tissue engineering. *International Journal of Biological Macromolecules* 2020;154:18–24.
35. Atoufi Z., Zarrintaj P., Motlagh G.H., Amiri A., Bagher Z., Kamrava S.K. A novel bio electro active alginate-aniline tetramer/agarose scaffold for tissue engineering:

Synthesis, characterization, drug release and cell culture study. *Journal of Biomaterials Science, Polymer* 2017;15:1617–1638.
36. Diez-Pascual A.M., Diez-Vicente A.L. Wound healing bionanocomposites based on castor oil polymeric films reinforced with chitosan-modified ZnO nanoparticles. *Biomacromolecules* 2015;16:2631–2644.
37. Díez-Pascual A.M., Xu C.P., Luque R. Development and characterization of novel poly(ether ether ketone)/ZnO bionanocomposites. *Journal of Materials Chemistry B* 2014;2:3065–3078.
38. Amalraj A., Haponiuk J.T., Thomas S., Gopi S. Preparation, characterization and antimicrobial activity of polyvinyl alcohol/gum arabic/chitosan composite films incorporated with black pepper essential oil and ginger essential oil. *International Journal of Biological Macromolecules* 2020;151:366–375.
39. Rakmai J., Cheirsilp B., Mejuto J.C., Torrado-Agrasar A., Simal-Gandara J. Physicochemical characterization and evaluation of bio-efficacies of black pepper essential oil encapsulated in hydroxypropyl-betacyclodextrin. *Food Hydrocolloids* 2017;65: 157–164.
40. Haider A., Haider S., Kang I.K., Kumar A., Kummara M.R., Kamal T., Han S.S., A novel use of cellulose based filter paper containing silvernanoparticles for its potential application as wound dressing agent. *International Journal of Biological Macromolecules* 2018;108:455–461.
41. Alavi M., Rai M. Recent advances in antibacterial applications of metal nanoparticles (MNPs) and metal nanocomposites (MNCs) against multidrug-resistant (MDR) bacteria. *Expert Review of Anti-infective Therapy* 2019;17(6):419–428.
42. Sathiyaseelan A., Shajahan A., Kalaichelvan P.T., Kaviyarasan V. Fungal chitosan based nanocomposites sponges—An alternative medicine for wound dressing. *International Journal of Biological Macromolecules* 2017;104:1905–1915.
43. Cahú T.B., Silva R.A., Silva R.P.F., Silva M.M., Arruda I.R.S., Silva J.F., Costa R.M.P.B., Santos S.D., Nader H.B., Bezerra R.S. Evaluation of chitosan-based films containing gelatin, Chondroitin 4-sulfate and ZnO for wound healing. *Applied Biochemistry and Biotechnology* 2017;183:765–777. doi:10.1007/s12010-017-2462-z.
44. Kim, M.S., Park, S.J., Gu, B.K., Kim C.H. Fabrication of chitosan nanofibers scaffolds with small amount gelatin for enhanced cell viability. *Applied Mechanics and Materials* 2015;749:220–224.
45. Sergi R., Bellucci D., Salvatori R., Cannillo V. Chitosan-based bioactive glass gauze: Microstructural properties, in vitro bioactivity, and biological tests. *Materials* 2020;13(-12):2819. doi:10.3390/ma13122819.
46. Luz, G.M., Mano, J.F. Chitosan/bioactive glass nanoparticles composites for biomedical applications. *Biomedical Materials* 2012;7(5):054104.
47. Xia G., Zhai D., Sun Y., Hou L., Guo X., Wang L., Li Z., Wang F. Preparation of a novel asymmetric wettable chitosan-based sponge and its role in promoting chronic wound healing. *Carbohydrate Polymers* 2020;227:115296. doi:10.1016/j.carbpol.2019.115296.
48. Mohanty C., Das M., Sahoo S.K. Sustained wound healing activity of curcumin loaded oleic acid based polymeric bandage in a rat model. *Molecular Pharmaceutics* 2012;9(10):2801–2811.
49. Negi P., Sharma G., Verma C., Garg P., Rathore C., Kulshrestha S., Lal U.R., Gupta B., Pathania D. Novel thymoquinone loaded chitosan-lecithin micelles for effective wound healing: Development, characterization, and preclinical evaluation. *Carbohydrate Polymers* 2020;230:115659.
50. Mariod A.A., Ibrahim R.M., Ismail M., Ismail N. Antioxidant activity and phenolic content of phenolic rich fractions obtained from black cumin (Nigella sativa) seedcake. *Food Chemistry* 2009;116:306–312.

51. Ehterami A., Salehi M., Farzamfar S., Samadian H., Vaez A., Ghorbani S., Ai J., Sahrapeyma H. Chitosan/alginate hydrogels containing Alpha-tocopherol for wound healing in rat model. *Journal of Drug Delivery Science and Technology* 2019;51:204–213.
52. Biesalski H.K. Polyphenols and inflammation: basic interactions. *Current Opinion in Clinical Nutrition & Metabolic Care* 2007;10(6):724–728.
53. Augustine R., Dan P., Schlachet I., Rouxel D., Menu P., Sosnik A. Chitosan ascorbate hydrogel improves water uptake capacity and cell adhesion of electrospun poly(epsilon-caprolactone) membranes. *International Journal of Pharmaceutics* 2019;559:420–426.
54. Wang X., Jia H.C., Feng Y.M., Hong L.H. Chitosan-ascorbate for periodontal tissue healing and regeneration in a rat periodontitis model: An effectiveness validation. *Journal of Clinical Rehabilitative Tissue Engineering Research* 2010;14(12):2268–2272.
55. Zhu L., Liu X., Du L., Jin Y. Preparation of asiaticoside-loaded coaxially electrospinning nanofibers and their effect on deep partial-thickness burn injury. *Biomedicine & Pharmacotherapy* 2016;83:33–40.
56. Qiu J., Yu L., Zhang X., Wu Q., Wang D., Wang X., Xia C., Feng H. Asiaticoside attenuates lipopolysaccharide-induced acute lung injury via down-regulation of NF-kB signaling pathway. *International Immunopharmacology* 2015;26:181–187.
57. Zhao X., Li P., Guo B., Ma P.X. Antibacterial and conductive injectable hydrogels based on quaternized chitosan-graft-polyaniline/oxidized dextran for tissue engineering. *Acta Biomaterialia* 2015;26:236–248.
58. Jun I., Jeong S., Shin H. The stimulation of myoblast differentiation by electrically conductive sub-micron fibers. *Biomaterials* 2009;30:2038–2047.
59. Qu J., Zhao X., Liang Y., Zhang T., Ma P.X., Guo B. Antibacterial adhesive injectable hydrogels with rapid self-healing, extensibility and compressibility as wound dressing for joints skin wound healing. *Biomaterials* 2018;183:185–199.
60. Zhang B., He J., Shi M., Liang Y., Guo B. Injectable self-healing supramolecular hydrogels with conductivity and photo-thermal antibacterial activity to enhance complete skin regeneration. *Chemical Engineering Journal* 2020;400:125994.
61. Song R., Zheng J., Liu Y., Tan Y., Yang Z., Song X., Yang S., Fan R., Zhang Y., Wang Y. A natural cordycepin/chitosan complex hydrogel with outstanding self-healable and wound healing properties. *International Journal of Biological Macromolecules* 2019;134:91–99.
62. Sundaram M.N., Amirthalingam S., Mony U., Varma P.K., Jayakumar R. Injectable chitosan-nano bioglass composite hemostatic hydrogel for effective bleeding control. *International Journal of Biological Macromolecules* 2019;129:936–943.
63. Ostomel T.A., Shi Q., Stucky G.D. Oxide hemostatic activity. *Journal of the American Chemical Society* 2006;128:8384–8385.
64. Tao F., Cheng Y., Shi X., Zheng H., Du Y., Xiang W., Deng H. Applications of chitin and chitosan nanofibers in bone regenerative engineering. *Carbohydrate Polymers* 2020;230:115658. doi:10.1016/j.carbpol.2019.115658.
65. Zhang M., Matinlinna J.P., Tsoi J.K.H., Liu W., Cui X., Lu W.W., Pan H. Recent developments in biomaterials for long-bone segmental defect reconstruction: A narrative overview. *Journal of Orthopaedic Translation* 2020;22: 26–33.
66. Shokri S., Movahedi B., Rafieinia M., Salehi H. A new approach to fabrication of Cs/BG/CNT nanocomposite scaffold towards bone tissue engineering and evaluation of its properties. *Applied Surface Science* 2015;357:1758–1764.
67. Jones J.R. Acta biomaterialia review of bioactive glass: From Hench to hybrids. *Acta Biomaterialia* 2013;9:4457–4486.
68. Nazemi K., Azadpour P., Moztarzadeh F., Urbanska A.M., Mozafari M. Tissue-engineered chitosan/bioactive glass bone scaffolds integrated with PLGA nanoparticles: A therapeutic design for on-demand drug delivery. *Materials Letters* 2015;138:16–20.

69. Jalali N., Moztarzadeh F., Mozafari M., Asgari S., Motevalian M., Alhosseini S.N. Surface modification of poly(lactide-co-glycolide) nanoparticles by D-α-tocopheryl polyethylene glycol 1000 succinate as potential carrier for the delivery of drugs to the brain. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 2011;392(1):335–342.
70. Pourhaghgouy M., Zamanian A., Shahrezaee M., Masouleh M.P. Physicochemical properties and bioactivity of freeze-cast chitosan nanocomposite scaffolds reinforced with bioactive glass. *Materials Science and Engineering C* 2016;58:180–186.
71. Zhang S., Prabhakaran M.P., Qin X., Ramakrishna S. Biocomposite scaffolds for bone regeneration: Role of chitosan and hydroxyapatite within poly-3-hydroxybutyrate-co-3-hydroxyvalerate on mechanical properties and in vitro evaluation. *Journal of the Mechanical Behavior of Biomedical Materials* 2015;51:88–98.
72. Zhao D., Cai L., Wu J., Li M., Liu H., Han J., Zhou J., Xiang H. Improving polyhydroxyalkanoate production by knocking out the genes involved in exopolysaccharide biosynthesis in Haloferax mediterranei. *Applied Microbiology and Biotechnology* 2013;97:3027–3036.
73. Maji K., Dasgupta S., Kundu B., Bissoyi A. Development of gelatin-chitosan-hydroxyapatite based bioactive bone scaffold with controlled pore size and mechanical strength. *Journal of Biomaterials Science, Polymer Edition* 2015;26(16):1190–1209.
74. Kim H.L., Jung G.Y., Yoon J.H., Han J.S., Park Y.J., Kim D.G., Zhang M., Kim D.J. Preparation and characterization of nano-sized hydroxyapatite/alginate/chitosan composite scaffolds for bone tissue engineering. *Materials Science and Engineering C* 2015;54:20–25.
75. Acevedo C.A., Olguín Y., Briceño M., Forero J.C., Osses N., Díaz-Calderón P., Jaques A., Ortiza R. Design of a biodegradable UV-irradiated gelatin-chitosan/nanocomposed membrane with osteogenic ability for application in bone regeneration. *Materials Science and Engineering C* 2019;99:875–886.
76. Teimouri A., Ebrahimi R., Emadi R., Beni B.H., Chermahini A.N. Nano-composite of silk fibroin–chitosan/Nano ZrO_2 for tissue engineering applications: Fabrication and morphology. *International Journal of Biological Macromolecules* 2015;76:292–302.
77. Pattnaik S., Nethala S., Tripathi A., Saravanan S., Moorthi A., Selvamurugan N. Chitosan scaffolds containing silicon dioxide and zirconia nano particles for bone tissue engineering. *International Journal of Biological Macromolecules* 2011;49:1167–1172.
78. Zhou Y., Xu L., Zhang X., Zhao Y., Wei S., Zhai M. Radiation synthesis of gelatin/CM-chitosan/β-tricalcium phosphate composite scaffold for bone tissue engineering. *Materials Science and Engineering C* 2012;32(4):994–1000.
79. Yaszemski M.J., Payne R.G., Hayes W.C., Langer R., Mikos A.G. Evolution of bone transplantation: Molecular, cellular and tissue strategies to engineer human bone. *Biomaterials* 1996;17:175–185.
80. Serra I.R., Fradique R., Vallejo M.C.S., Correia T.R., Miguel S.P., Correia I.J. Production and characterization of chitosan/gelatin/β-TCP scaffolds for improved bone tissue regeneration. *Materials Science and Engineering C* 2015;55:592–604.
81. Puvaneswary S., Talebian S., Raghavendran H.B., Muralia M.R., Mehrali M., Afifi A.M., Kasim N.H.B.A., Kamarul T. Fabrication and in vitro biological activity of βTCP-Chitosan-Fucoidan composite for bone tissue engineering. *Carbohydrate Polymers* 2015;134:799–807.
82. Park S.J., Lee K.W., Lim D.S., Lee S. The sulfated polysaccharide fucoidan stimulates osteogenic differentiation of human adipose-derived stem cells. *Stem Cells Development*, 2012;21(12):2204–2211.
83. Vacanti J.P., Vacanti C.A., Lanza R.P., Langer R., Vacanti J. *Principles of Tissue Engineering*, Second ed., Academic Press, Cambridge, MA, 2000, pp. 3–9.
84. Langer R., Vacanti J.P., Tissue engineering. *Science* 1993;260:920–926.

85. Roseti L., Parisi V., Petretta M., Cavallo C., Desando G., Bartolotti I., Grigolo B. Scaffolds for bone tissue engineering: State of the art and new perspectives. *Materials Science and Engineering C* 2017;78:1246–1262.
86. Thavornyutikarn B., Chantarapanich N., Sitthiseripratip K., Thouas G.A., Chen Q., Bone tissue engineering scaffolding: Computer-aided scaffolding techniques. *Progress in Biomaterials* 2014;3:61–102.
87. Gravel M., Vago R., Tabrizian M. Use of natural coralline biomaterials as reinforcing and gas-forming agent for developing novel hybrid biomatrices: Microarchitectural and mechanical studies. *Tissue Engineering* 2006;12(3):589–600.
88. Gravel M., Gross T., Vago R., Tabrizian M. Responses of mesenchymal stem cell to chitosan–coralline composites microstructured using coralline as gas forming agent. *Biomaterials* 2006;27:1899–1906.
89. Matassi F., Nistri L., Paez D.C., Innocenti M. New biomaterials for bone regeneration. *Clinical Cases in Mineral and Bone Metabolism* 2011;8(1):21–24.
90. Kolanthai E., Sindu P.A., Khajuria D.K., Veerla S.C., Kuppuswamy D., Catalani L.H., Mahapatra D.R. Graphene oxide-a tool for the preparation of chemically crosslinking free alginate−chitosan−collagen scaffolds for bone tissue engineering. *ACS Applied Materials Interfaces* 2018;10:12441–12452.
91. Zhou T., Li G., Lin S., Tian T., Ma Q., Zhang Q., Shi S., Xue C., Ma W., Cai X. Electrospun poly (3-Hydroxybutyrate-Co-4-Hydroxybutyrate)/Graphene oxide scaffold: Enhanced properties and promoted in vivo bone repair in rats. *ACS Applied Materials Interfaces* 2017;9:42589–42600.
92. Demir A.K., Elçin A.E., Elçin Y.M. Strontium-modified chitosan/montmorillonite composites as bone tissue engineering scaffold. *Materials Science and Engineering C* 2018;89:8–14.
93. Aguzzi C., Cerezo P., Viseras C., Caramella C. Use of clays as drug delivery systems: Possibilities and limitations. *Applied Clay Science* 2007;36:22–36.
94. Bonnelye E., Chabadel A., Saltel F., Jurdic P. Dual effect of strontium ranelate: Stimulation of osteoblast differentiation and inhibition of osteoclast formation and resorption in-vitro. *Bone* 2008;42:129–138.
95. Pineda-Castillo S., Bernal-Ballén A., Bernal-López C., Segura-Puello H., Nieto-Mosquera D., Villamil-Ballesteros A., Muñoz-Forero D., Munster L. Synthesis and characterization of poly(Vinyl Alcohol)-chitosan-hydroxyapatite scaffolds: A promising alternative for bone tissue regeneration. *Molecules* 2018;23:2414. doi:10.3390/molecules 23102414.
96. Mi Zo S., Singh D., Kumar A., Cho Y.W., Oh T.H., Han S.S. Chitosan-hydroxyapatite macroporous matrix for bone tissue engineering. *Current Science* 2012;102:1438–1446.
97. Peng X., Hu M., Liao F., Yang F., Ke Q., Guo Y., Zhu Z., La-Doped mesoporous calcium silicate/chitosan scaffolds for bone tissue engineering. *Biomaterials Science* 2019;7:1565–1573.
98. Shahsavari R., Hwang S.H. Size- and shape-controlled synthesis of calcium silicate particles enables self-assembly and enhanced mechanical and durability properties. *Langmuir* 2018;34:12154–12166.
99. Shi D., Shen J., Zhang Z., Sci C., Chen M., Gu Y., Liu Y. Preparation and properties of dopamine-modified alginate/chitosan–hydroxyapatite scaffolds with gradient structure for bone tissue engineering. *Journal of Biomedical Materials Research Part A* 2019;107(8):1615–1627. doi:10.1002/jbm.a.36678.
100. Sadeghinia A., Davaran S., Salehi R., Jamalpoor Z. Nano-hydroxy apatite/chitosan/gelatin scaffolds enriched by a combination of platelet-rich plasma and fibrin glue enhance proliferation and differentiation of seeded human dental pulp stem cells. *Biomedicine & Pharmacotherapy* 2019;109:1924–1931.

101. Kokubo T., Kim H.M., Kawashita M. Novel bioactive materials with different mechanical properties. *Biomaterials* 2003;24(13):2161–2175.
102. Cao H., Kuboyama N. A biodegradable porous composite scaffold of PGA/beta-TCP for bone tissue engineering. *Bone* 2010;46:386–395.
103. Jamalpoor Z., Mirzadeh H., Joghataei M.T., Zeini D., Bagheri-Khoulenjani S., Nourani M.R. Fabrication of cancellous biomimetic chitosan-based nanocomposite scaffolds applying a combinational method for bone tissue engineering. *Journal of Biomedical Materials Research Part A* 2015:103A:1882–1892.
104. Ruixin L., Cheng X., Yingjie L., Hao L., Caihong S., Weihua S., Weining A., Yinghai Y., Xiaoli Q., Yunqiang X., Xizheng Z., Hui L. Degradation behavior and compatibility of micro, nanoHA/chitosanscaffolds with interconnected spherical macropores. *International Journal of Biological Macromolecules* 2017;103:385–394.
105. Wang Y., Qian J., Zhao Z., Liu T., Xu W., Suo A. Novel hydroxyethyl chitosan/cellulose scaffolds with bubble-likeporous structure for bone tissue engineering. *Carbohydrate Polymers* 2017;167:44–51.
106. Zhang T., Zhang H., Zhang L., Jia, S. Liu J., Xiong Z., Sun W. Biomimetic design and fabrication of multilayered osteochondral scaffolds by low-temperature deposition manufacturing and thermal-induced phase-separation techniques. *Biofabrication* 2017;9:025021.
107. Rahman S., Rana M., Spitzhorn L.S., Akhtar N., Hasan Z., Choudhury N., Fehm T., Czernuszka J.T., Adjaye J., Asaduzzaman S.M. Fabrication of biocompatible porous scaffolds based on hydroxyapatite/collagen/chitosan composite for restoration of defected maxillofacial mandible bone. *Progress in Biomaterials* 2019;8:137–154.
108. Erickson A.E., Sun J., Levengood S.K.L., Swanson S., Chang F.C., Tsao C.T., Zhang M. Chitosan-based composite bilayer scaffold as an in vitro osteochondral defect regeneration model. *Biomedical Microdevices* 2019;21:34.
109. Cima M.J., Sachs E., Cima L.G., Yoo J., Khanuja S., Borland S., Wu B., Giordano R. Computer derived microstructures by 3D printing: bio-and structural materials. *International Solid Freeform Fabrication Symposium Proc: DTIC Doc* 1994;181–190.
110. Chia H.N., Wu B.M. Recent advances in 3D printing of biomaterials. *Journal of Biological Engineering* 2015;9:4. doi:10.1186/s13036-015-0001-4.
111. Yang Y., Chu L., Yang S., Zhang H., Qin L., Guillaume O., Eglin D., Richards R.G., Tang T. Dual-functional 3D-printed composite scaffold for inhibiting bacterial infection and promoting bone regeneration in infected bone defect models. *Acta Biomaterialia* 2018;79:265–275.
112. Dong L., Wang S.J., Zhao X.R., Zhu Y.F., Yu J.K. 3D- printed poly(ε-caprolactone) scaffold integrated with cell-laden chitosan hydrogels for bone tissue engineering. *Scientific Reports* 2017;7:13412. doi:10.1038/s41598-017-13838-7.
113. Tsai C.H., Hung C.H., Kuo C.N., Chen C.Y., Peng Y.N., Shie M.Y. Improved bioactivity of 3D printed porous titanium alloy scaffold with chitosan/magnesium-calcium silicate composite for orthopaedic applications. *Materials* 2019;12:203. doi:10.3390/ma12020203.
114. Chen S., Shi Y., Zhang X., Ma J. Biomimetic synthesis of Mg-substituted hydroxyapatite nanocomposites and three-dimensional printing of composite scaffolds for bone regeneration. *Journal of Biomedical Materials Research* 2019;107:2512–2521.
115. Chen T., Zou Q., Du C., Wang C., Li Y., Fu B. Biodegradable 3D printed HA/CMCS/PDA scaffold for repairing lacunar bone defect. *Materials Science & Engineering C* 2020;116:111148.
116. Liu Y., Ai K., Lu L. Polydopamine and its derivative materials: Synthesis and promising applications in energy, environmental, and biomedical fields. *Chemical Reviews* 2014;114(9):5057–5115.

117. Jing X., Mi H.Y., Peng J., Peng X.F., Turng L.S. Electrospun aligned poly(propylene carbonate) microfibers with chitosan nanofibers as tissue engineering scaffolds. *Carbohydrate Polymers* 2015;117:941–949.
118. Venkatesan J., Anil S., Kim S.K., Shim M.S. Chitosan as a vehicle for growth factor delivery: Various preparations and their applications in bone tissue regeneration. *International Journal of Biological Macromolecules* 2017;104:1383–1397.
119. Even J., Eskander M., Kang J. Bone morphogenetic protein in spine surgery: Current and future uses. *Journal of the American Academy of Orthopaedic Surgeons* 2012;20:547–552.
120. Sobhani A., Rafienia M., Ahmadian M., Naimi-Jamal M.R. Fabrication and characterization of polyphosphazene/calcium phosphate scaffolds containing chitosan microspheres for sustained release of bone morphogenetic protein 2 in bone tissue engineering. *Tissue Engineering and Regenerative Medicine* 2017:14(5):525–538.
121. Bastami F., Paknejad Z., Jafari M., Salehi M., Rad M.R., Khojasteh A. Fabrication of a three-dimensional β-tricalcium-phosphate/gelatin containing chitosan-based nanoparticles for sustained release of bone morphogenetic protein-2: Implication for bone tissue engineering. *Materials Science and Engineering C* 2017;72:481–491.
122. Deng N., Sun J., Li Y., Chen L., Chen C., Wu Y., Wang Z., Li L. Experimental study of rhBMP-2 chitosan nano-sustained release carrierloaded PLGA/nHA scaffolds to construct mandibular tissue-engineered bone. *Archives of Oral Biology* 2019;102:16–25.
123. Tong S., Xu D.P., Liu Z.M., Du Y., Wang X.K. Synthesis of and *in vitro* and *in vivo* evaluation of a novel TGF-β1-SF-CS three-dimensional scaffold for bone tissue engineering. *International Journal of Molecular Medicine* 2016;38:367–380.
124. Baylink D.J., Finkelman R.D., Mohan S. Growth factors to stimulate bone formation. *Journal of Bone and Mineral Research* 1993;8(suppl 2):S565–S572.
125. Oryan A., Alidadi S., Bigham-Sadegh A., Moshiri A., Kamali A. Effectiveness of tissue engineered chitosan-gelatin composite scaffold loaded with human platelet gel in regeneration of critical sized radial bone defect in rat. *Journal of Controlled Release* 2017;254:65–74.
126. Intini G. The use of platelet-rich plasma in bone reconstruction therapy. *Biomaterials* 2009;30:4956–4966.
127. Liao H.T., Tsai M.J., Brahmayya M., Chen J.P. Bone regeneration using adipose-derived stem cells in injectable thermo-gelling hydrogel scaffold containing platelet-rich plasma and biphasic calcium phosphate. *International Journal of Molecular Sciences* 2018;19:2537. doi:10.3390/ijms19092537.
128. Wang B., Guo Y., Chen X., Zeng C., Hu Q., Yin W., Li W., Xie H., Zhang B., Huang X., Yu F. Nanoparticle-modified chitosan-agarose-gelatin scaffold for sustained release of SDF-1 and BMP-2. *International Journal of Nanomedicine* 2018;13:7395–7408.
129. Dou D.D., Zhou G., Liu H.W., Zhang J., Liu M.L., Xiao X.F., Fei J.J., Guan X.L., Fan Y.B. Sequential releasing of VEGF and BMP-2 in hydroxyapatite collagen scaffolds for bone tissue engineering: Design and characterization. *International Journal of Biological Macromolecules* 2019;123:622–628.
130. Krishnan L., Priddy L.B., Esancy C., Klosterhoff B.S., Stevens H.Y., Tran L., Guldberg R.E. Delivery vehicle effects on bone regeneration and heterotopic ossification induced by high dose BMP-2. *Acta Biomaterialia* 2017;49:101–112.
131. Choi B.H., Zhu S.J., Kim B.Y., Huh J.Y., Lee S.H., Jung J.H. Effect of plateletrich plasma (PRP) concentration on the viability and proliferation of alveolar bone cells: an in vitro study. *International Journal of Oral and Maxillofacial Surgery* 2005;34(4):420–424.
132. Nihouannen D.L., Saffarzadeh A., Gauthier O., Moreau F., Pilet P., Spaethe R., Layrolle P., Daculsi G. Bone tissue formation in sheep muscles induced by a biphasic calcium phosphate ceramic and fibrin glue composite. *Journal of Materials Science: Materials in Medicine* 2008;19(2):667–675.