Chitosan: A Promising Scaffold biomaterial in 3D Bone Tissue Engineering and its biological activitiess

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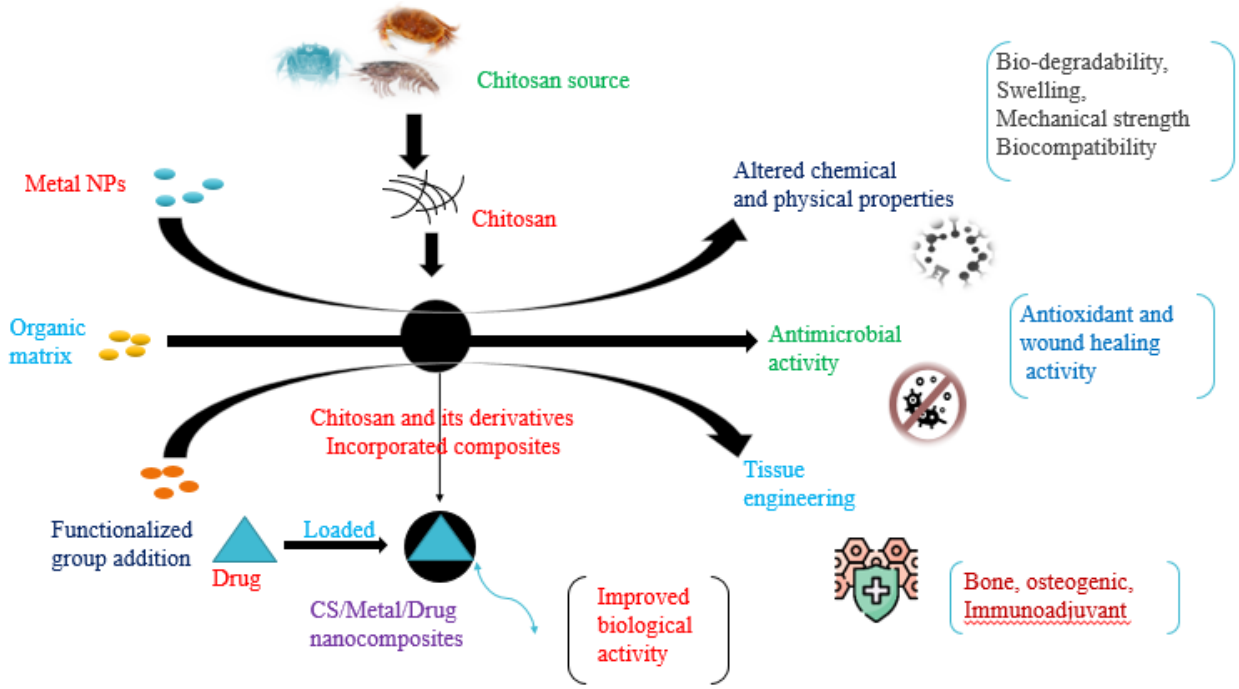
Abstract The ability to mimic the microenvironment of the human body through fabrication of scaffolds itself a great achievement in the biomedical field. However, the search for the ideal scaffold is still in its infant stage and there are significant challenges to overcome. In the modern era, scientific communities are more attracted to natural substances due to their excess biological ability, low cost, biodegradability, and lesser toxic than synthetic lab made products. Chitosan is a well-known polysaccharide that has recently grabbed high amount of attention for its biological activities, especially in 3D bone tissue engineering (BTE). Chitosan greatly matches with the native tissues and thus stands out as a popular candidate for bioprinting. This review focuses on the potential of chitosan based scaffolds advancement and the drawbacks in bone treatment. Chitosan-based nanocomposites have exhibited strong mechanical strength, water-trapping ability, cellular interaction, and biodegradability characteristics. Chitosan derivatives have also encouraged and provided different routes of treatment and enhanced biological activities. 3D tailored bioprinting have opened new doors to design and manufacture scaffolds of biological, mechanical, and topographical properties.

GRAPHICAL ABSTRACT

The applications of chitosan-based scaffolds in critical-sized bone defects have developed an increasing interest in recent years. This review exploits chitosan's various biochemical properties in bone tissue regeneration. A diagrammatic illustration is given:



ABBREVIATIONS

BTE bone tissue engineering, GAGs glycosaminoglycan, Hap hydroxyapatite, ECM extracellular matrix, CAD computer-assisted design, AM additive manufacturing, FDM fused deposition method, LTDM low temperature deposition manufacturing, PHBV/CaSH Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)/calcium sulfate hemihydrates, CMC carboxymethyl Chitosan, TMC N-trimethylchitosan, PLGA poly lactic-co-glycolic acid, TCP tricalciumphosphate, NPs nanoparticles.

1. Introduction

Bone tissues being dynamic in nature, carries the intrinsic capability to regenerate themselves in response to any damage or injury[1]. It goes under continuous renewal and remodeling throughout its adult life Figure1. Nevertheless, the reconstruction of critical-sized bone damage remains a key challenge. For the past few years, the global aging population has been tremendously in search of advanced bone substitutes. A case study by Global

Burden of Disease has revealed that a total of 336.5 million prevalent cases, and 74,000 deaths are caused by musculoskeletal disorders such as arthritis and back pain[2]. Other bone-related defects are caused by either trauma, abnormal growth, or tumor[3]. Another common skeletal disorder gathering attention is osteoporosis (OP), in which bones become so fragile and brittle that it results in fractures. In 2015, OP alone caused 1.3 million fracture in US and 2.33 million fracture in China in 2010. It has been estimated that the numbers will exponentially rise in China and will reach up to 5.99 million in 2050[4]. Unfortunately, due to all these setbacks, the economy and the health of citizens across the world are being compromised. To overcome this challenge, clinical treatments have been widely used such as bone cements, bone grafting which includes autografts and allografts[5]. However, these treatments appeared with various limitations which mainly include the short lifespan of grafts, donor site morbidity, infections, immune rejection, and limited donors[3]. In recent years, there is a breakthrough in regeneration of bone tissue through tissue-engineering technology called bone tissue engineering (BTE)[6]. Advances in BTE can achieve regeneration through the construction of biodegradable porous scaffolds Figure 2. Regeneration of bone tissues requires interplay between three components, scaffold material, growth factors that induce osteogenesis and angiogenesis, and the cells undergoing osteogenic differentiation[7]. Three-dimensional (3D) bioprinting has shown significant potential in tissue engineering to regenerate impaired tissues due to its ability to create tissue-engineered scaffolds with aesthetic benefits in terms of structures over other traditional scaffold fabrication techniques[8]. Meskinfam et al. produced unique approach to characterize polymeric scaffolds for bone tissue engineering. The study characterized various pathways, needed for BTE, concentrating on scaffold aspects such as morphology, architecture, structural properties, and surface chemistry[9].

Chitosan is one of the popular natural polysaccharides that has been studied over time due to its tunable chemical and biological properties and is widely used in BTE applications. Chitosan stands out as an appealing bone scaffold material as it promotes osteoblast cell adhesion and proliferation, as well as the creation of a mineralized bone matrix[10]. Research findings show that there are some drawbacks related to the chitosan scaffold like water

insolubility, and weak mechanical strength, nevertheless when combined with other polymers or ceramics material improves chitosan in bone healing applications[11]. On the positive note, chitosan tremendously resembles glycosaminoglycans (GAGs) found in the extracellular matrix (ECM) contributing to its non-toxic biocompatible nature. Thus, chitosan presents new avenues for bone treatments and stands out competitively as a versatile biopolymer in terms of its bioavailability, low production cost, and ability to elicit cell adherence, mineralization, and neovascularization [12].

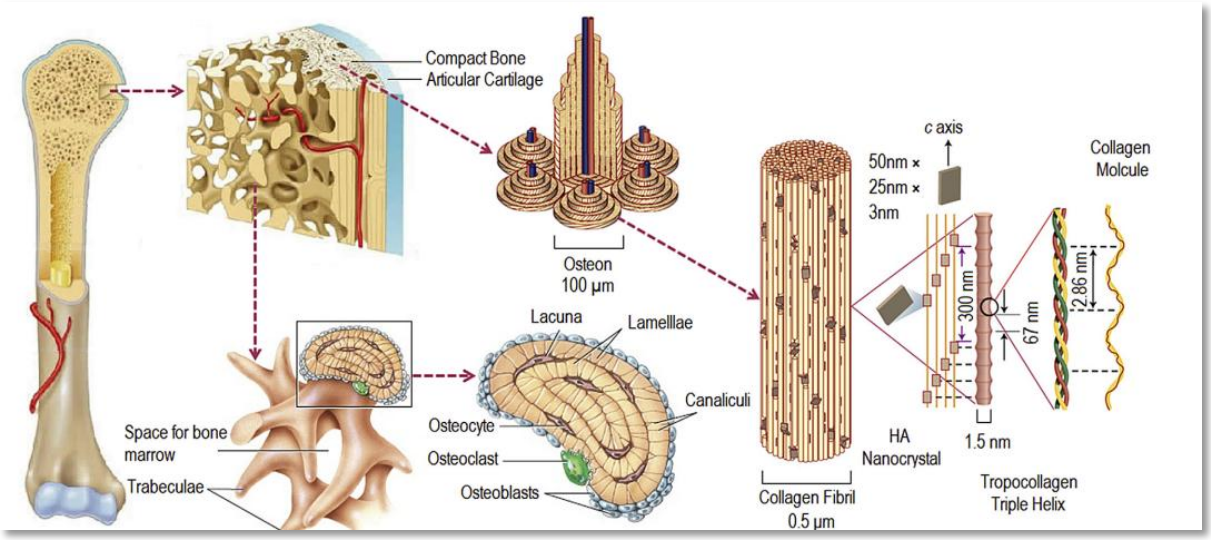


Figure 1. Anatomical structure of bone. The organic matrix of osteoid is composed of collagen fibers, ground substances immersed in semisolid gel. The bone cells that produce organic matrix are known as osteoblasts, the bone cells that break down old bones are called osteoclasts, and mature osteoblasts that can no longer form new bones are called osteocytes. Source: Reprinted with permission from [13]

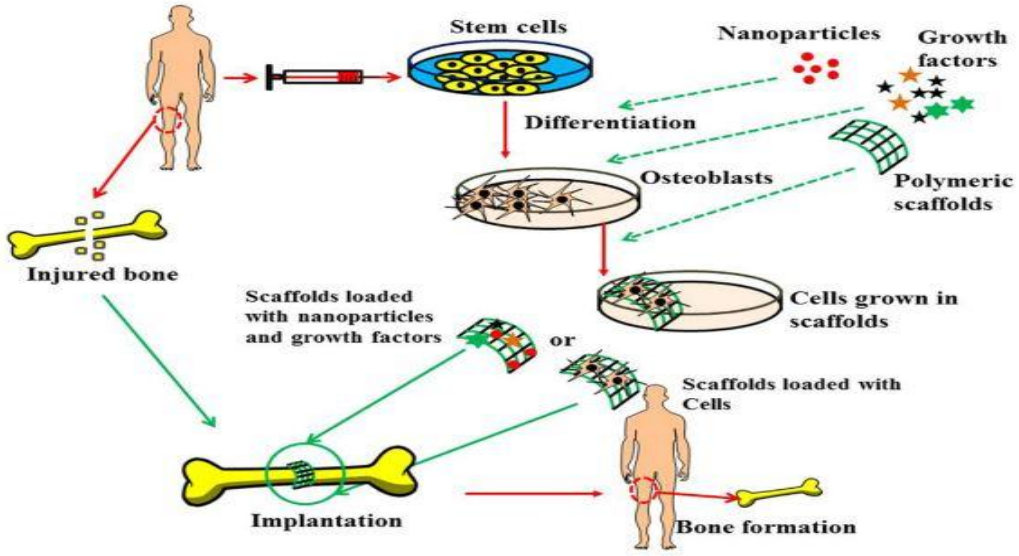


Figure 2. Bone tissue engineering process and scaffold implantation. Collected stem cells with different nanoparticles and growth factors are grown with in scaffold and incorporates the scaffold loaded cells in the defected area for regeneration. Source: Reprinted with permission from [1].

1.1. Source and physio-chemical property of Chitosan

Lengths of the polymer chain, deacetylation degree, and molecular weight are some major parameters which are required to determine the physical, chemical as well as biological properties of the chitosan scaffold. Beside these key parameters, there are other secondary criteria such as viscosity, crystallinity, coagulation, solubility. So, each year, almost 100 billion tons of chitins are being produced by crustaceans, mollusks, fungi, and other related organisms. Chitin stands in the second position in terms of abundance after cellulose[14] and in terms of structure, it is a linear polysaccharide, made up of (1,4)-linked N-acetyl-d-glucosamine units. At the industrial level, chitin is extracted from its shell in basic three steps, deprotonation of chitin by adding an alkaline solution, demineralization

in which it is further treated with an acidic media, and lastly discoloration[15]. From being available in nature in huge amounts to having exceptional features such as biodegradability, biocompatibility, raw chitin severely lacks behind when it comes to solubility. Henceforth, it makes us all shift towards chitosan, a derivative of chitin. The conversion of chitin into chitosan can be enzymatic or through a chemical process. The chemical process is widely preferred as the production expense is low and it is more suitable for mass production[16]. The reaction through which chitosan is obtained is chitin deacetylation where chitin is treated with a strong sodium hydroxide solution (40-50%) at 100 ℃ or higher to remove some or all the acetyl group and further the N-Acetyl-D-glucosamine and D-glucosamine repeating units linked by β-(1-4) glycoside bonds are randomly placed Figure 3. More precisely, when the degree of deacetylation is up to 60%, it becomes soluble in acidic environment. The amino groups of chitosan protonate in the acidic media (pH below 4) and becomes cationic in nature thus attributing it to interact with a variety of molecules and making it the only cationic polysaccharide found in marines[17]. Moreover, chitosan resembles GAGs found in the ECM contributing to its non-toxic biocompatible nature. The physiochemical characteristics of chitosan like biodegradability, solubility, crystallinity are all greatly influenced by the degree of deacetylation[18]. The electrostatic interaction between the negatively charged bacterial cell envelope and positively charged chitosan is responsible for its antimicrobial activity too. Apart from its antimicrobial and antioxidant activity, it is also used in wound healing which has been approved by FDA, the Food and Drug Administration[18]. Chitosan shows better bioactivity when the molecular mass is less than 20kDa. Again viscosity is strongly related to the molecular weight of chitosan. In the aqueous acid medium, as the solvent temperature increases, viscosity decreases. Chitosan also possesses excellent coagulating properties. In presence of N_2 or any metal ion, the amino group present in chitosan acts as an electron donor and is responsible for metal ion chelation. The majority of these characteristics are related to the chitosan's backbone's free protonable amino groups, which also contribute to its solubility in acidic solution. Additionally, chitosan biopolymer may be physically altered, opening up a number of shape possibilities. As a result, this polysaccharide is utilized in a number of industries, including tissue engineering, drug delivery systems, and also for the treatment of cancer.

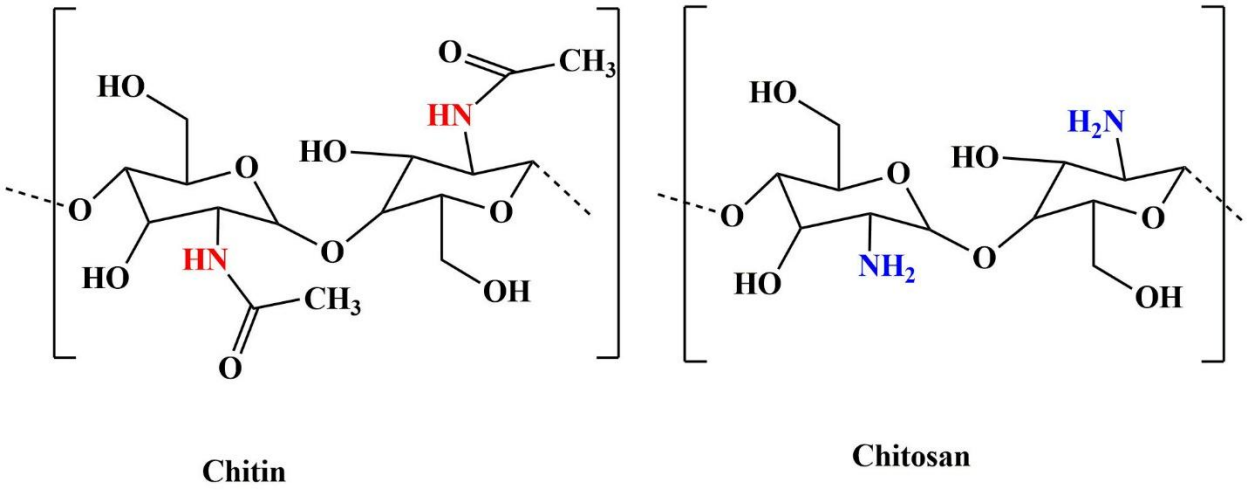


Figure 3. Schematic representation of Chitin deacetylation to produce chitosan.

1.2. Chitosan in bone tissue engineering

Reyna-Urrutia et al. has reported and provided remarkable remarks on BTE that bone regeneration is an urgent therapeutic need for the treatment of bone abnormalities produced by a variety of reasons. Studies have reported clinical demand for chitosan-based scaffolds which provide flexibility in scaffold production, allowing for effective advances in BTE. Chitosan has received a lot of interest in recent years for its usage as a graft material in BTE, either alone or in combination with other materials Figure 4. Porosity, biocompatibility, water retention, protein adsorption, mechanical strength, biomineralization, and biodegradability are all desirable features in scaffolds for BTE applications. This review focuses on chitosan and its characteristics, as well as the role of chitosan and other polymeric and ceramic materials as scaffolds for bone tissue healing applications[1]. Synthesized biocomposites CS-CL-PVA-HA, which are composed of chitosan (CS), hydroxyapatite (HA), caprolactone (CL), and polyvinyl alcohol (PVA) evaluate against osteogenic activities. The CS-CL-PVA-HA scaffold demonstrated good cell survival, proliferation, and differentiation of DPSCs, indicating its potential for in vitro and in vivo bone defect research. The

inclusion of HA improved its osteoconductive characteristics, allowing for more effective bone tissue regeneration[19] Figure 5.

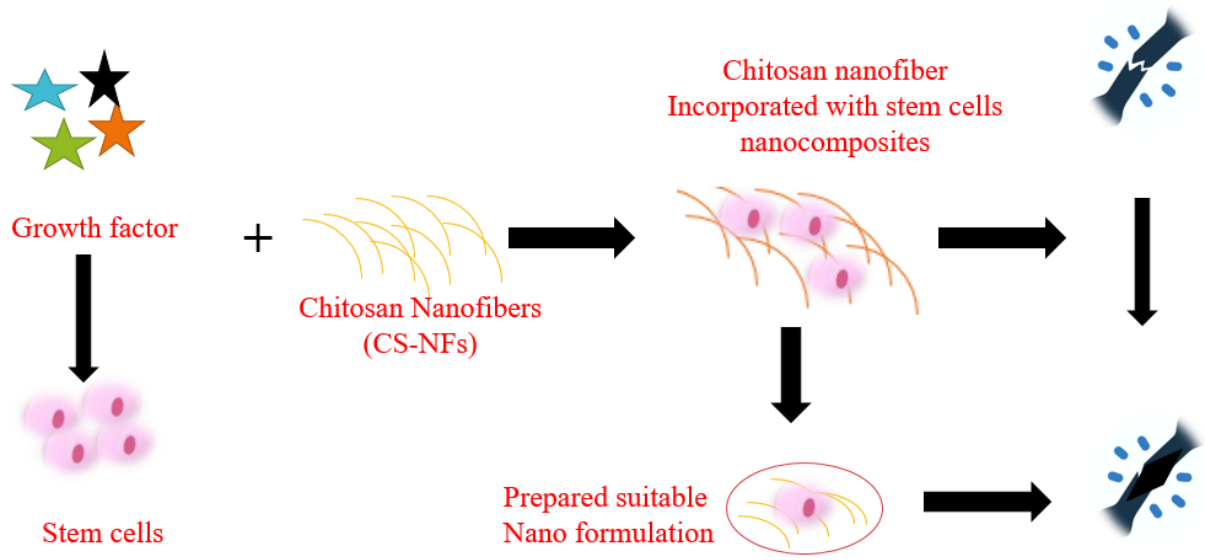


Figure 4. Synthesizing chitosan-based different substances, which are used in bone treatments. Combining chitosan nanofibers with various growth factor infused within stem cell could heal the damaged area.

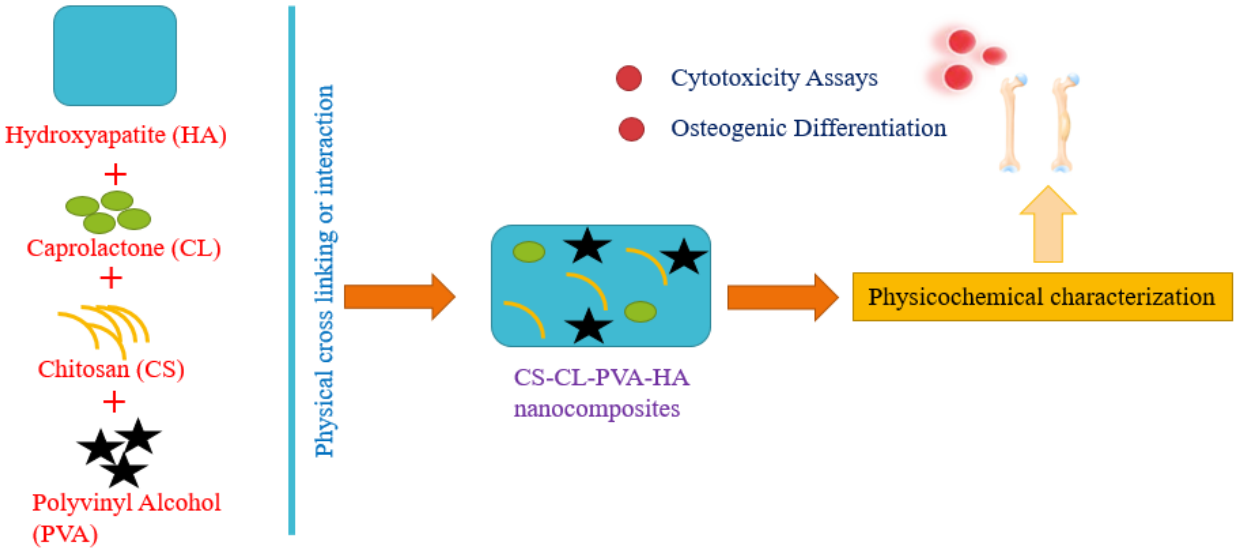


Figure 5. Synthesis as well biological activities of biocomposite (CS-HA-PVA-CL) and through their interactions, the nanocomposite cause osteogenic differentiation and regeneration.

Kim et al. describes chitosan biopolymer possess numerous properties which makes it appealing for biological applications and produced into a wide range of structures, including nanoparticles, scaffolds, hydrogels, and membranes, all of which can be tuned to achieve a desired result. In vivo regeneration and repair of numerous tissues and organs, including but not limited to bone, cartilage, dental, skin, nerve, cardiac, and other tissues, have been proven using composite chitosan-based biomaterials. De novo tissue creation, resident stem cell differentiation, and extracellular matrix rebuilding were found in chitosan-based formulations treated preclinical models of various tissue lesions[20] Figure 6. An invitro study using poly (lactic acid)/chitosan based nanocomposite provided better mechanical stability and enhanced the production of calcium phosphate without any toxicity to the neighboring cells[21]. In another study, chitosan/polyvinyl alcohol mesh in β tricalcium phosphate aerogel demonstrated effective bone renewal and proved that the following scaffold mesh could be the popular bone substitute candidate in biomedical engineering[22].

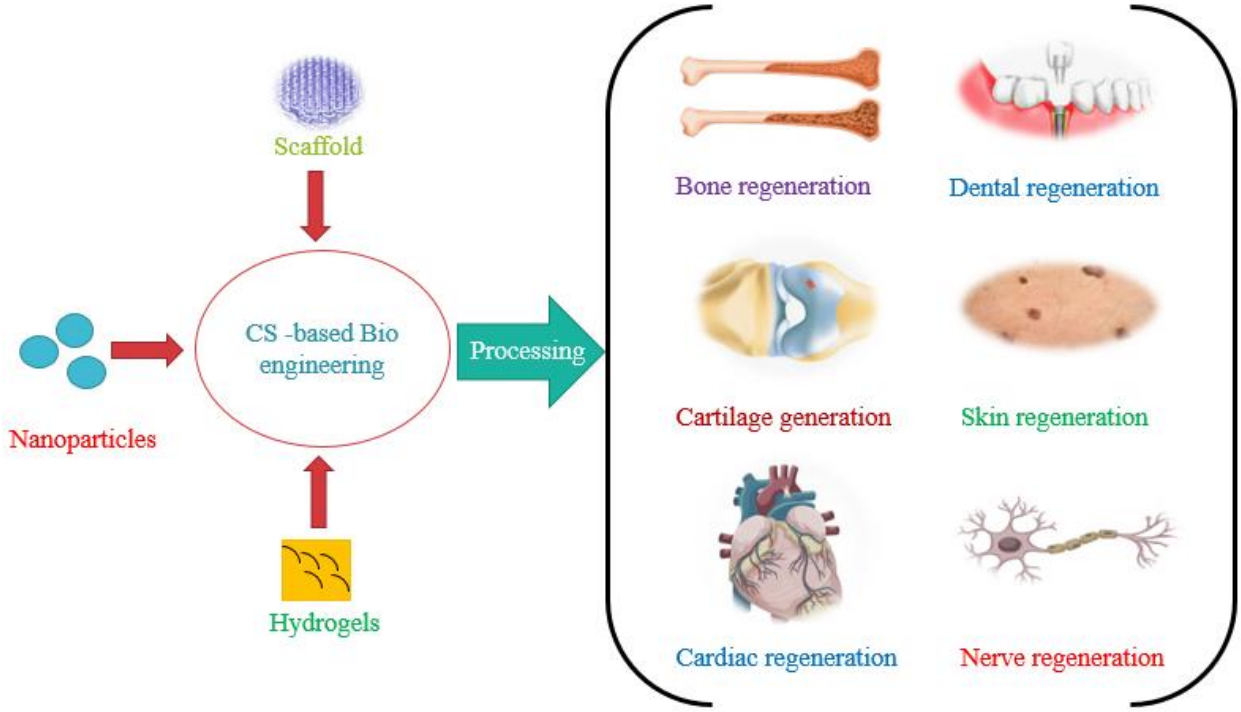


Figure 6. Various chitosan-based formulations, utilized in several area regenerations according to the need.

1.3. Chitosan biomaterial in Cartilage Regeneration

Injury to articular cartilages which holds the synovial joints results in premature arthritis and poor lifestyle if left untreated. The chondrocytes do not undergo mitosis and does not contain any blood vessels and thus the capacity of cartilage to regenerate is very low[23]. The available clinical treatment like chondrocyte implantation has several drawbacks such as unable to completely fill the injured site, it does not guarantee formation of hyaline cartilage and furthermore it does not integrate the repaired tissue into native tissue[24]. Henceforth, alternative treatment using bone tissue engineering and regenerative medicines are of huge interest. Based on the past research, materials infused with chitosan has shown positive outcomes and due to its antimicrobial and biodegradable properties it has proven to be one of the best alternative substance in cartilage engineering. The akin structure of ECM of N-acetylglucosamine groups of chitosan and ECM of cartilage attributes enhanced chondrogenesis[25]. Chitosan based

hydrogels due to their ability of fill the injury site in any conceivable way and high water holding capacity showed great potential in clinical studies and composite hydrogels can be manipulated to have suitable mechanical and structural properties to further enhance cell proliferation and differentiation[26]. In an experiment, while delivering the adipose derived MSCs, the chitosan/dibenzaldehyde-terminated PEG hydrogel provided suitable microenvironment for cell proliferation by facilitating proper supply of oxygen and nutrients. Further extended the experiment for better cytocompatibility and mechanical property, silanized hydroxypropylmethyl cellulose and silanized chitosan based hydrogel managed to prove the secretory activity and viability of adipose derived stem cell when transplanted the hydrogel into the subcutis of nude mice[27]. When the same gel injected into a canine, it showed osteochondral regeneration irrespective of adipose derived stem cell. Thus, it depicts that the composition of hydrogel is capable of repairing osteochondral damages[27]. Biphasic chitosan scaffold in osteochondral engineering provides the scaffold to compose numerous microenvironment on various levels and using the same feature, chitosan bilayered scaffold were used and buccal fat pad stem cell which were hypoxic were seeded in the same scaffold. The scaffold then expressed proteoglycans and reinforced COL2 thus proving regeneration of hyaline cartilage[28]. Another study using the bilayered chitosan scaffold with a hydroxyapatite porous bone layer and a thick cartilage layer. Further, magnesium and copper ions were integrated in the bone and cartilage layers respectively. Results demonstrated that Cu^{2+} ions promoted osteogenesis via enhanced expression of VEGF and Mg^{2+} showed cartilage regeneration. The bone layer of the scaffold provides the scaffold mechanical strength by forming the subchondral bone and also upregulated the migration of new cartilage to move towards the synovial joint[29]. 3D tailored chitosan/poly(ε-caprolactone) hybrid scaffold embedded with synovial MSCs and tetrahedral nucleic acid framework demonstrated invivo regeneration. The scaffold upregulated the production of ECM components of cartilage and also showed differentiation of synovial MSCs as the positive chitosan recruited the negatively charged nucleic acid[30]. Also, chitosan could be used as a multilayer scaffold for therapeutics release. In a study, multilayer chitosan/polycaprolactone scaffold within conjugated kartogenin enhanced the chondrogenesis of MSCs[31].

1.4. Chitosan derivatives

Despite having numerous biomedical applications of chitosan alone or as a composite with other biomaterials and ceramic particles, the use is still limited. Disadvantage is chitosan solubility in aqueous solutions is dependent on pH and at neutral pH the solubility of chitosan is limited. Moreover, presence of glycosidic linkage makes it easily degradable in the body due to abundance of hydrolytic cleavage of lysozyme [32]. Hence, the biocompatible nature of chitosan can be maximized when it is modified to a certain extent aiding its benefit in solubility and degradation properties. Table 1 comprises various modified chitosan derivatives based scaffolds.

1.4.1. Carboxymethyl chitosan

To enhance the solubility of chitosan, functional groups are introduced and the addition of carboxyalkyl groups is one of them. Carboxymethyl chitosan (CMC) is obtained through a reaction at the C6 hydroxyl group or the NH_2 group and the products are either N-CMC, O-CMC, or N-O-CMC. It is amphoteric and thus widely used in medical fields Figure 7. CMC polymer can show strong bioactivity when loaded with hydrophobic drugs [33]. CMC depends on the degree of substitution, and this further depends on molecular weight and amount of carboxylate agent. Molecular weight and degree of substitution are inversely proportional to each other[34]. When the carboxyl group of CMC is compared with other biopolymers, it greatly resembles alginate and agarose. Both are highly compatible

with CMC. Improvement in the tensile strength was observed when CMC was cross-linked with alginate and agarose in neural stem cell differentiation[35]. The effect of CMC is also investigated in Alzheimer's disease. It proved that invitro CMC delayed the release of galanthamine and nanoceria[36]. CMC also increased the efficacy of compounds showing poor bioavailability and solubility [37]. The advantages of using CMC are as follows; improves adhesion and proliferation and osteoblasts, enhances mechanical strength, enhances osteoinductivity, and promotes new bone formation[38]. CMCS is also popularly active in pharmaceutical fields due to its antibacterial, antiviral, antitumor and lipid lowering properties. Carboxymethyl chitosan/PVA electrospun scaffold showed hMSC adherance and proliferation with no cytotoxicity [39]. Injectable gels of CMC–gelatin–nHAp enhanced the proliferation of osteoblasts and illustrates no inflammation at the site of implantation [40]. Carboxymethyl chitosan/gelatin/β-TCP composites produced through ultrasonic radiation showed better porous in structure along with optimum mechanical strength. The micro-CT showed the scaffold promoted biocompatibility and bone formation when implanted in the mandibular region of a canine model [32].

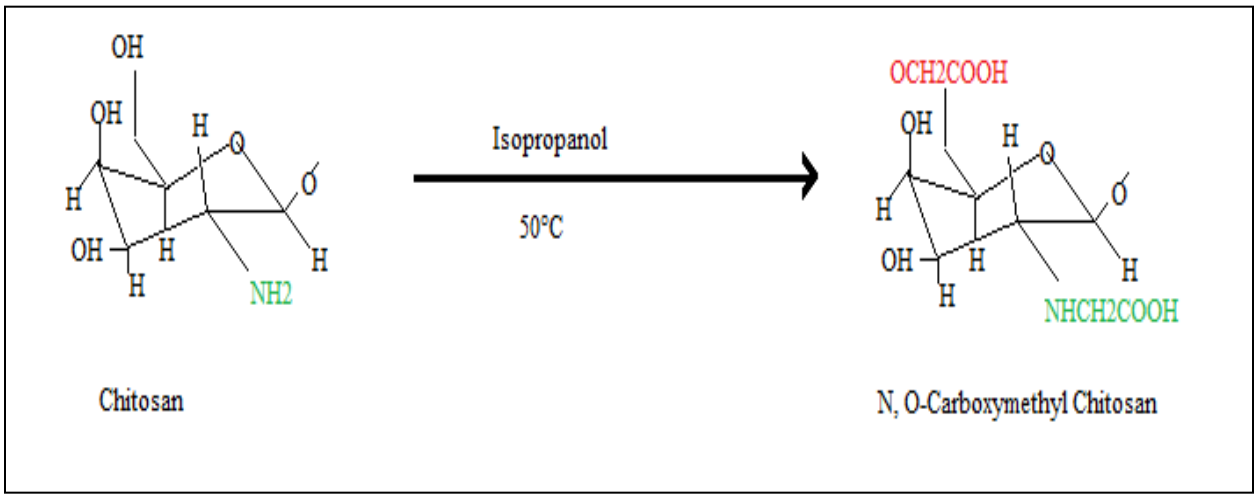


Figure 7. Schematic presentation of synthesis of CMC from chitosan in the presence of isopropanol.

1.4.2. Acylated chitosan

Another common modification is the acylation of chitosan. Here multiple acyl groups (e.g. aliphatic and aromatic) are added to the chitosan chain and the reaction is carried out with various organic acids Figure 8. The acylation disrupts the inter- and intramolecular hydrogen bonding and increases the solubility level. Further, there are 2 types of acylation: N-acylation, and O-acylation. In N-acylation, the amide is formed by the C2-NH2 reaction, and in O-acylation, the ester is formed in a reaction of C6-OH[41, 42]. O- acylated chitosan are lipid-soluble and can dissolve in chloroform and can be used for enhancing biomaterial stability[33]. Whereas N-acylated chitosan shows anti-coagulability, better biocompatibility, anti-inflammatory reactions and widely used as carrier in biomedical applications.

Figure 8. Schematic presentation of synthesis of N-acylated chitosan from chitosan.

1.4.3. Quaternary ammonium chitosan

The quaternary ammonium chitosan derivative is not only water-soluble but also a positively charged hydrophilic polymer. pH under 6.5, chitosan without any modifications is positively charged whereas quaternized chitosan remains positive above 6.5 pH which makes it a better derivative in terms of anti-bacterial activity. The reaction for the formation of quaternary ammonium chitosan takes place in C2–NH2 and the alkyl group is added in place of an amino group. N-trimethyl chitosan (TMC), the strongest muco-adhesive polymer is one of the products of the quaternized chitosan Figure 9. The quaternized chitosan proved to be better in terms of antimicrobial, biodegradable,

and non-toxic properties[43]. A case study of TMC/heparin multilayer on cortical bone allograft showed similar behavior of periosteum, a fibrous covering present on the bone surface[44]. Anti-bacterial properties of TMC have also been studied and found that it hampers the growth of both gram-positive and gram-negative bacteria [45]. Quaternary ammonium chitosan also shows non-toxic, mucoadhesive properties and used in medicine. Due to its antibacterial property it is used a filler and anti-inflammatory drug.

Figure 9. Schematic presentation of synthesis of TMC from chitosan.

1.4.4. Thiolated chitosan

Again, a great modification of chitosan where it displays not only hydration but also in situ gelling capability. The reaction occurs between the thiol-containing coupling agent and a primary amino group of the chitosan. The thiol group present readily adheres with the cysteine present in mucus through the formation of a covalent bond and thus

it displays better mucosal adhesion properties [46] Figure 10. Methacrylamide chitosan derivative can also be thiolated. In tissue engineering, thiolated chitosan is widely known for delivering growth factors like VEGF, and PDGF for blood vessel regeneration. It also acted as a thermosensitive cell carrier for tissue regeneration. Other applications include biodegradable bandages in wound treatment, anti-microbial coatings in polymer films, As^{3+}/As^{5+} removal from groundwater in wastewater treatment, in cosmetics to prevent contact dermatitis [47].

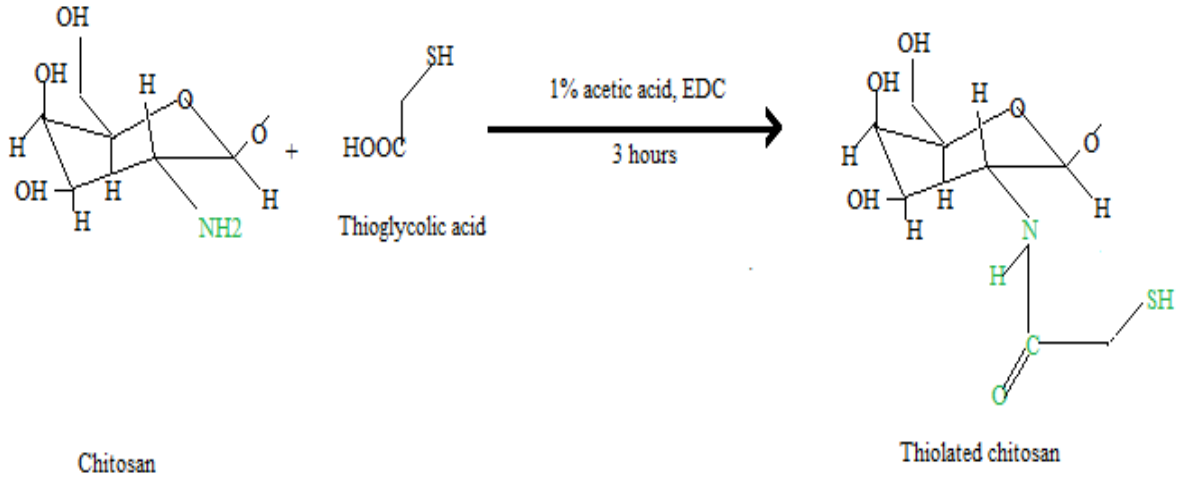


Figure 10. Schematic presentation of synthesis of thiolated chitosan.

Table 1. Some common derivatives of Chitosan and their features.

Modified Chitosan	Composite	In vitro studies	Animal defect area and model	Characteristics	Ref.
Methacrylate chitosan	Chitosan-β-glycerol phosphate salt/lithium phenyl-2,4,6-trimethylbenzoylphosphinate(LAP) photoinitiator	cell proliferation observed in all cell lines	_	3D hydrogel presented better adherence and migrating cells confirmed the cell amiability and permeability	[37]

Carboxymethyl chitosan	Polydopamine (PDA)/ Hap	Biocompatible and better cell adhesion of MC3T3-E1 mouse preosteoblastic cells	Male New Zealand white rabbits, femoral bone defect	3D scaffolds achieved a satisfactory degree of biodegradability and balanced bone rejuvenation and promoted cancellous bone defect repair.	[48]
N, O-carboxymethyl chitosan	Polyphosphate	Induced Saos-2 cell mineralization	6-week-old male SD rats, calvarial defect	Bone biomineralization and in vivo studies demonstrated significant regenerative-inducing activity.	[49]
Quaternized chitosan	PLGA/Hap	Human bone marrow-derived MSCs proliferated and differentiated towards the osteoblast lineage	6-week-old male SD rats and dorsum subcutaneous implantation model	Exerted anti-bacterial effect and displayed strong tissue integration. Neovascularization upon in vivo implantation.	[50]
Quaternized chitosan	PLGA/Hap	_	Female SD rats: femoral shaft defect; female New Zealand white rabbits: condyle defect	Demonstrated significant anti-infective effect and bone-repairing ability in both the two bone defect models.	[51]
Phosphorylated chitosan/ Disodium (1 → 4)-2- deoxy-2-sulfoaminoβ-Dglucopyranuronan chitosan	PLGA/TCP	_	_	Exerted remarkable mechanical properties and absorbability	[52]

Phosphorylated chitosan/ Disodium (1 → 4)-2- deoxy-2-sulfoaminoβ-Dglucopyranuronan chitosan	PLGA/TCP	_	Mature female New Zealand white rabbits, critical-sized ulnar bone defect	Possessed dual functions, including osteogenic and Osteoclastic properties	[53]

2. Mechanical and biological properties of 3D Bio-printed Chitosan scaffold

2.1. Porosity

Proper pore dimensions are very crucial in scaffold fabrication as it supports cell infiltration, cell adhesion, secretion of different ECM components, and tissue growth. Numerous studies highlightes various optimal pore sizes, 20-100m is beneficial for cell infiltration whereas >100m improves neovascularization. It has also been reported that improper pore size indirectly affects cell behavior as small pores limits cell permeability and large pores limit ligand availability for cell-to-cell binding. Polymer concentration, printing technique, cross linkers, temperature, and added number of nanoparticles (NPs) are basic parameters for pore designing chitosan scaffold. Genipin,ethyl-3[3-dimethylaminopropyl]carbodiimide hydrochloride(EDAC), and tripolyphosphate (TPP) are some common cross linker that improves the morphology and interconnectivity of the pores as well as of scaffold[54].

2.2. Mechanical property

The Young's modulus and compressive strength of cortical bone are 15–20 GPa and 100–200 MPa respectively, whereas cancellous bone possesses 0.1–2 GPa and 2–20 MPa, respectively[55]. Studies have shown a positive relationship between the stiffness of a scaffold and the behavior of cells nearby. It influences the mesenchymal stem cells (MSC) to differentiate into specific cell types. Hence, the scaffolds must possess an appropriate load-bearing capacity. Chitosan on the other hand has a moderate strength which limits its use. However, adding ceramics or other polymers with the chitosan increases the tensile strength of the scaffold[56]. Namely, the addition of graphene (GR) within the chitosan scaffold enhances the strength of the scaffold. Another 3D bio-printed GR/chitosan/Gel/hyaluronic acid scaffold influences the MSCs to proliferate[57]. The incorporation of gelatin and tricalcium phosphate also can accelerate the strength of the scaffold by up to 70%[58]. Wu et al. [59] investigated the tensile strength and cell viability of chitosan hydrogel observed under SEM and optical microscopy. For testing stress, the hydrogel went seven times under various processes such as neutralization, and non-neutralization and finally was tested using Insight MTS50KN (electromechanical machine). For cell movement, L929 fibroblast cells were seeded on the hydrogel. The results illustrated that compared to the printed filament, the post-neutralized scaffold's tensile property was improved due to complex inter and intra-molecular hydrogen bond formation. The material was also tested using tweezers and was stretched for one minute and then released. Here also complete recovery of the filament was observed. SEM imaging also displayed excellent fibroblast growth on the hydrogel demonstrating chitosan as a suitable scaffold to multiply on.

2.3. Osteogenesis and vascularization activity

Chitoan scaffolds lacking vascular networks results in cellular apoptosis and to overcome a novel strategy has been invented. The RGD peptide sequence i.e., Arg-Gly-Asp embedded in CaP/chitosan porous scaffold containing MSCs was implanted in the rat's radial defect bone. The introduction promoted neovascularization along with osteoblastic proliferation[60].3D printed allyl chitosan also induces osteogenesis when hMSCs were cultured at an early stage[61]. The magnesium calcium silicate (Mg–CLS/chitosan-coated) titanium alloy (Ti–6Al–4V) scaffold

elicited surface hydrophilicity, which aided in the adherence, proliferation, and differentiation of hMSCs. And a study revealed that a high percentage of new bone was formed in Mg–CLS/ chitosan scaffold when evaluated histomorphometrically in femur defects of rabbits [62]. An attempt was done by fabricating CaP lattice-shaped 3D scaffold using chitosan as a binding agent and an extrusion-based technique was used. The results showed high expression of ALP, OCN, and COL1[63]. Likewise, another attempt was carried out using Mg/nHAp/chitosan/gel scaffold and demonstrated that the osteoblast marker genes like Runx2, OCN, and COL1 were high. The attaching and differentiating capacity of osteogenetic cells also greatly increased[64]. New bone formation in calvarial defects was also investigated when osteoblast-like cells were gained from TCP/Col/ chitosan hydrogel[65]. Chitosan/gelatin scaffold showed the formation of extracellular matrix in femur implantation in an orthotopic mouse model. Moreover, the preosteoblastic cells and bone marrow stem cell successfully adhered and proliferated in the chitosan/gelatin scaffold and showed high expression of osteogentic markers such as ALP, RUNX2. Membrane chitosan/polycaprolactone with strontium and calcium phosphate nanoparticles resulted angiogenesis by releasing vascular endothelial growth factor and also formed calcified nodules thus proving the membrane capability to osteogenic and angiogenic differentiation. Chitosan/alginate multilayer film fabricated on interleukin 4 loaded on chitosan/alginate multilayer film provided sustainable release of the cytokinin and enhanced the angiogenic markers[66].

2.4. Swelling and Biodegradation

In in-vivo, chitosan is mainly degraded by lysozyme found in every mammalian tissue (serum, saliva, other fluids) whereas, in vitro; chitosan can be degraded via hydrolysis or oxidation. The biodegradation rate is directly proportional to the crystallinity, molecular mass, and length of the chain. Degraded chitosan releases

monosaccharides which either infuse with the metabolic pathway of GAG or excrete [67]. Demonstrated chitosan degradation in rats where chitosan-based hydrogels were injected in the back and 19 days no traces of gel were found and was completely resorbed. Pure collagen and chitosan/collagen hydrogels swelling ratios and degradation showed collagen alone had a maximal swelling rate which disturbed the geometry of the scaffold. However, when chitosan was infused in collagen, it maintained its integrity by reducing the swelling rate. The chitosan addition thus proved its significant effect on retaining collagen. The degradation performance was also tested for the same scaffold. In the presence of collagenase type I, pure collagen completely degraded in an hour at room temperature in a 100rpm shaker. The addition of chitosan significantly decreased the shrinking of 3D scaffolds. The tension strength of the collagen/chitosan scaffold was also enhanced after an increase in the chitosan ratio. The addition of chitosan in the collagen/chitosan scaffold also has improved the rigidity of the scaffold[68]. The structural morphology of gelatin/alginate hydrogel was also revived by the addition of carboxymethyl chitosan (CMCS). Moreover, the degradation rate of the hydrogel was also slowed down by CMCS[69].

2.5. Immunoadjuvant property

In response to the tumor, chitosan biologically exhibits the ability to activate macrophages to produce nitric oxide (NO) and interleukin 1 (IL-1). It also acquires to respond to chemical stimuli like neutrophils[70]. 3D- chitosan sponges also revealed that a higher rate of deacetylation results in an increase in osteopontin (OPN), IL-6, vascular endothelial growth factor-A (VEGF), alkaline phosphatase (AP), and lowering the degree of deacetylation maximized the secretion of sclerostin (SOST) and osteoprotegerin[71].

2.6. Antioxidant and antibacterial activity

Structurally, chitosan is a cationic polysaccharide consisting of amino groups (NH_2) and becomes soluble under pH level 6.5. Their positive charge/ functional active amino group is responsible for the antimicrobial activity of chitosan, making it interesting for biomedical applications. Experimentally proven, chitosan inhibits proliferation of

various bacteria, fungi and yeasts, with different mechanisms, not are not all fully clarified. One of the simplest mechanism of action involves electrostatic interactions between the NH_3^+ sites of chitosan which is positively charged and the membranes of microbial cells (negatively charged). The interaction disrupts the permeability of the microbial cell, causing the release of intracellular material. Chitosan also sometimes interacts with peptidoglycan and altering the osmotic gradient of the membrane wall.

It has been revealed that 0.02% of chitosan in vitro has positive antioxidant effects such as reducing lipid peroxidation and enhancing glutathione peroxidase (GSH-PX), superoxide dismutase (SOD), catalase (CAT) antioxidant enzymes. Higher the deacetylation, the better the antimicrobial effect[72]. The incorporation of CMCS in gel/alg hydrogel also aided antibacterial activity and provided better mechanical stability to the scaffolding[69]. Photocured chitosan-Ph hydrogel (phenolic hydroxyl moiety) demonstrated suppressed bacterial growth when *E.coli* (Gram-negative bacteria) and *S. aureus* (Gram-positive bacteria) were tested for antimicrobial activity.

For bone reconstruction, chitosan scaffolds can be used solely with bioactive molecules, or they can be combined with other materials such as ceramics, synthetic, or any other natural polymers. It blends with other polymers perfectly and with proper adjustments, chitosan scaffolds show positive effects in medical fields in Table 2.

Table 2. Chitosan-based 3D scaffolds and their characteristics for bone tissue reconstruction.

Polymers	Preparation methods	Animal model and defect areas	Comments	Ref.
chitosan/silk fibroin/cellulose nanoparticle	BP	Calvarial defect in a rat model	The osteo-immunomodulatory potential of the printed 3D scaffold was analyzed in hBMSCs and revealed improved bone formation. Hydrogel also showed better recovery strength	[73]

Chitosan-Vanillin-Bioglass	BP	Inbred C57BL/6NHsd female mice (6 weeks, ENVIGO)	The mechanical property and bioactivity were improved in the scaffold, and in vivo studies also revealed better anti-bacterial property and osteoconductivity in vivo, data showed scaffold-supported BMP2-induced ectopic ossicle formation in mice.	[74]
gelatin/chitosan/polyvinyl alcohol/nano-hydroxyapatite(GCPH)	_	Four-week-old Sprague-Dawley rats	The GCPH scaffolds exhibited improved properties in terms of strength, adherence, osteogenic potential, porosity, proliferation, biodegradation	[75]
Regenerated cellulose nanofiber(Rcl)-chitosan	_	_	Hydrogel demonstrated better biomineralization and enhanced viability of pre-osteoblast cell (MC3T3-E1) was also enhanced	[76]
Microchannel chitosan	Leaching method and freeze-drying	Six male 12-week-old Sprague-Dawley (SD) rats	Microchannel architecture scaffolds showed better cell survival, better proliferation, and migration in vitro, and encouraged tissue ingrowth in vivo	[77]
Chitosan/Gel/Hap	FDM	_	scaffolds exhibited good porosity and Oxygen plasma treated scaffold exhibited enhanced roughness, wettability, and higher MC3T3-E1 cell proliferation	[78]
PLA/β-TCP/CP-amoxicillin	BP	_	Effective antimicrobial property, highest biocompatibility, structural stability, cell viability, proper porosity, and compressed strength was exhibited	[79]
Mg–CLS/Chitosan coated Ti–6Al–4V	Selective Laser Melting	New Zealand rabbits-femoral bone defects	Surface-modified scaffold showed better cellular functions such as cell adhesion, proliferation, and differentiation, and promoted osteogenesis and mineralization	[62]
PHBV/CaSH/ chitosan	FDM	Adult male Sprague Dawley (SD) rats - rat ectopic osteogenesis model	Increased rBMSCosteogenesis by upregulating the expression of osteogenic genes such as RUNX2, COL1, OCN, OPN, and BMP2	[80]

PLGA/nHAp/chitosan with rhBMP2	LTDM	13-week-old New Zealand white rabbits – mandibular bone defect	Sustained release of rhBMP2, biocompatibility in vitro, and 45.5% new bone formation in the defect site in vivo	[81]
PCL/chitosan	FDM	7-week-old male nude mice-subcutaneous implantation	Scaffold possessed high compressive strength and a favorable bone matrix formation and osteogenesis	[82]
BG/chitosan(NELL1) carrying BMSCs	Fiber Deposition Technology	Adult female rhesus monkeys- alveolar bone defect	model X-ray and micro-CT observations showed that the new bone was like the native bone in terms of mass, density, hardness, and shape	[83]
GR/Gel/Chitosan/ TCP	BP	_	Showed better antimicrobial property by the scaffold and alkaline phosphatase activity were also observed. Moreover, the printed material exhibited good osteogenic proliferation	[84]
Hap/Chitosan/ Genipin	Direct Ink Writing	_	Cyto-friendly environment, therefore, showing enhanced adhesion of human osteoblastic cells (MG63) to the scaffolds	[85]
Chitosan/PVA/HAp with BMP2	BP	_	The elastic modulus of the chitosan scaffold closely matches that of natural bone, good biocompatibility, improved MSC attachment, and proliferation	[86]
Chitosan/Bioglass 46S6 with infused gentamicin sulfate	Freeze Drying Technique	_	Scaffold exhibited better release kinetics of gentamicin sulfate invitro	[87]
Chitosan-Bioglass and carbon nanotube	Novel hot press and salt leaching process	_	Compressive strength nearly matched with the cancellous bone, better biodegradability, and Promoted attachment and proliferation of MG-63 cells	[88]
Chitosan/bioactive glass/ PLGA nanoparticles	Freeze Drying Technique	_	Mechanical strength was increased, and the swelling behavior of the scaffold was lowered	[89]

Chitosan-Fucoidan–Tri-calcium phosphate	Freeze Drying	_	Improved compression strength was improved and enhanced apatite deposition was observed	[90]
Alkali treated Chitosan	3D Freeze drying	_	Up to 97%, the scaffold showed inhibition against bacterial growth as well as able to produce hydroxyapatite in vitro	[91]
Ionic chitosan hydrogels (NaOH)	Extrusion-based	_	Simple preparation, self-assembly in an aqueous medium, low cytotoxicity	[92]

3. **Commercially available and ongoing clinical trials of Chitosan products**

As mentioned above that Chitosan is FDA approved and is widely preferred in biomedical areas. chitosan is not only limited to bone regeneration but also shows potential application in drug delivery, weight loss medication, hypocholesterolemic agent, hemodialysis, absorbable sutures.

BST-Gel®, manufacturer Piramal Healthcare Canada Ltd., is a chitosan-based self-forming hybrid composite. It is an injectable mineral polymer which comprises of two components- liquid components (water based thermal sensitive) containing chitosan and an organic mono-phosphate source and Ca^{2+}, F, $SrCO_3$ mineral component. These components are then mixed and formed into an injectable paste. In situ, after injecting the slurry gets converted into gel like substance. The product is utilized in bone filling, cartilage repair, restoring intervertebral disk[93]. Other products are summarized in Table3.

Table 3. List of Chitosan-based medications available and their applications[94].

NO.	Product	Manufacturer	Description	Application	Year of launch
1	BST-Gel®	Piramal Healthcare Canada Ltd.	Chitosan scaffold for cartilage repair	Chronic wound healing, intervertebral disc restoration, bone filling, cartilage repair	2015
2	HemconChitoGauze®	XR PRO Tricol Biomedical Inc	Hemostatic dressing	Wound dressing	2010
3	CELOX RAPID	–	Celox rapid hemostatic gauze works with just 60 s of compression	Quickest acting gauze, 60s compression stops the severe arterial bleeding	2011
4	SILVAPRO	–	an antibacterial burn dressing of size 5″ × 9″ is a combination of Chitosan and Ionic Silver.	In a single solution it gives dual cooling as well as antibacterial protection	–
5	Chitosan fibers	ChiPro GmbH.	Chitosan fibre	Textile or medical	2021
6	M-Chitosan	M-Chitosan	The fabric in the inner layer remains 99.9 % antibacterial even after 100 washes	masks are reusable and washable	–
7	Talymed®	Marine polymer technologies, Inc.	Sterile wound matrix	Wound dressing material	2015
8	Reaxon®	Medovent	Nerve guide	Nerve regeneration, hemostatic	2018
9	CELOX VASCULAR	–	2 × 2-inch sized hemostatic gauze patch	Local management of surface bleeding from vascular access sites, percutaneous catheters, or tubes.	2010

There are numerous clinical trials ongoing with the chitosan containing bioactive products for treatment of various disease, wound healing and many more Table4 (https://www. clinicaltrials.gov/ (as accessed on Feb 11, 2023).

Table 4. Ongoing clinical studies of various Chitosan based drugs in medical field.

No.	NTC number	Title	Status	Disease condition	Intervention	Outcome	Phase	Location
1	NCT02668055	Tb4 collagen and chitosan porous sponge scaffolds skin substitute treatment is difficult to heal wounds	Completed	Wounds	TB4	Wound regression	1	China
2	NCT05214807	Long-term safety and performance of Kiomedine CM-chitosan supplementation in advanced symptomatic osteoarthritis	Recruiting	knee Osteoarthritis	KiOmedine® CM-chitosan Synvisc-One®	knee pain improvement	4	Belgium
3.	NCT02081885	Tricalcium phosphate and chitosan as bone regenerator versus autologous graft in surgery for mandibular fracture	-	Mandibular Fractures	Chitosan Graft	Bone density	3	Mexico
4	NCT04365270	Antibacterial effect and clinical performance of chitosan modified glass ionomer	Completed	Dental caries	Chitosan glass ionomer	Antibacterial activity	3	Egypt
5	NCT01895933	Efficacy and safety of the investigational device, SurgiShield anti-adhesion barrier gel	Completed	Wound healing	5mL surgishield	Adhesion rate	1	Korea

6	NCT05333211	Ortho-R® for rotator cuff repair compared with standard of care rotator cuff repair without OrthoR®	Recruiting	Rotator cuff tears	Ortho-R/PRP	-	1 & 2	United States

4. Conclusion and future outlook

This review consolidates and provides insight into the past and the current trends being followed in BTE using chitosan-based scaffolds. Chitosan exhibits a promising role as a biomaterial for BTE. However, certain limitations must be taken care of. First, the temperature must be controlled in 3D printing since chitosan is not thermoplastic and can lead to degradation. A temperature above 220 ◦C limits the chitosan modification and results in its decomposition. Secondly, chitosan alone shows improper cell adhesion and requires simple alterations or modifications to enhance the adhering capability. Furthermore, solubility and mechanical properties also limit its application due to which it is blended with other polymers. Chitosan as a compound is insoluble in water as well as in most organic solvents, which limits both its usage and scope in biomedicine fields. However, chitosan containing active functional groups, chitosan could be modified through chemical reaction and various derivatives could be obtained. Chitosan derivatives are popular as it shows greater extend of solubility, pH-sensitive targeting and increased ways of delivery systems and are more in demands. The muco-adhesiveness, biodegradability, antimicrobial, and many more properties make chitosan a more attractive biopolymer than other materials. Additionally, derivatives of chitosan are more in demand, and the properties are further improved via chemical modifications. All together mixed with other materials and with bioactive compounds embedded, 3D bioprinting of chitosan biopolymer has achieved a new level and it is considered now a biopolymer of the future. In the coming years, we believe that cutting-edge technologies will lead to extensive new research and variations using chitosan and that will bring greater prospects in the biomedical field. Moreover, chitosan hydrogels are not only bound to

bone regeneration. They can also be constructed and applied in other fields of interest such as vascular regeneration, periodontal regeneration, cartilage, drug delivery, wound healing, and many more. Altogether, this review helps to broaden our understanding of the development of chitosan hydrogels for future clinical applications.

5. Challenges

Firstly, to understand the maximum potential of bioink, more knowledge in R&D is required. Bioinks are still in their infancy stage as there is not a single bioink material that fulfills the whole biological properties required for bioprinting. Secondly, although the 3D bioprinting method carries high potential in customizing desired products, the ability to control pore size, its distribution, printing scaffold, and cells even with complicated shapes, it still lacks in bio mimicking the whole structure and organ properly. Thirdly, the scarcity of animal models. Currently, most experimental models are healthy and there are few animals left with diseases to discuss with. Fourthly, transportation and storage of hydrogels as they are fragile and tend to get damaged very easily which creates problems in experimental procedures. Lastly, the rapid disintegration and mechanical property of the chitosan hydrogels is poor which hinders its other beneficial properties. If the above-mentioned concerns are properly addressed, then the clinical research of chitosan hydrogels in bone regeneration can be achieved with precision.

Conflicts of interest

The authors declare no conflict of interest.

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