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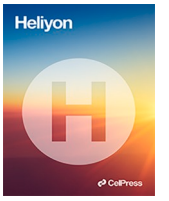

Review article

Recent advances of chitosan-based composite hydrogel materials in application of bone tissue engineering

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A B S T R A C T

Bone defects, stemming from trauma, tumors, infections, and congenital conditions, pose significant challenges in orthopedics. Although the body possesses innate mechanisms for bone self-repairing, factors such as aging, disease, and injury can impair these processes, jeopardizing skeletal integrity. Addressing substantial bone defects remains a global orthopedic concern, with variables like gender, lifestyle and preexisting conditions influencing fracture risk and complication rates. Traditional repair methods, mainly bone transplantation including autografts, allografts and xenografts, have shown effectiveness but also present limitations. Autologous bone grafts, highly valued for their osteogenic properties, require additional surgeries with extended hospitalization, and carry risks associated with the donor site. The development of advanced biomaterials offers promising new avenues for bone repair. An ideal material should exhibit a combination of biocompatibility, biodegradability, bone conduction, porosity, strength, and the ability to stimulate bone formation. Chitosan (CS), derived from chitin, stands out due to its biocompatibility, biodegradability, low immunogenicity, non-toxicity, and a wide range of biological activities, including antioxidant, anti-tumor, anti-inflammatory, antimicrobial, and immunomodulatory properties. Notably, CS has shown the properties to promote bone regeneration, increase bone density, and accelerate fracture healing. This review provides a comprehensive examination of CS-based hydrogels for bone repair aiming to inspire researchers by presenting new ideas for innovative CS-based solutions, thereby advancing their potential applications in the field of bone repair.

1. Introduction

1.1. Current status of bone tissue engineering

Among orthopedic diseases, bone defects have long been a disease of high prevalence [1], which are often caused by trauma, malignant tumors, infections, and congenital diseases [2]. Despite bone tissue's natural ability to self-repairing, factors such as aging, diseases, and injuries can significantly impair this process, jeopardizing the integrity of the skeletal system [3,4]. Research indicates that the probability of bone nonunion after bone defects ranges from 5 to 15 percent [5]. The challenge of regenerating new bone

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makes repairing significant bone defects a major orthopedic issue worldwide. Additionally, medical factors such as gender, lifestyle, and previous illnesses can also influence the risk of fractures and complications during the recovery process [6,7].

Autografts, allografts and xenografts, among other bone repair methods, are still considered the most effective methods for addressing bone defects. Autologous bone transplantation, in particular, is regarded as the gold standard for bone repair due to its excellent osteogenic capacity, cell integrity, and the ability to seamlessly integrate with the original bone tissue. However, recent research highlights its drawbacks, including the need for additional surgeries, extended hospitalization, and a rise of donor site morbidity [8–11]. The rapid development of biomaterials offers new solutions for bone defects [12,13]. An ideal material for bone repair should exhibit the benefits of biocompatibility and biodegradability, while also be demonstrated its bone conduction, high porosity, robust mechanical strength, and the capacity to efficiently stimulate new bone formation [14,15]. Early bone repair materials were non-biodegradable, such as metallic materials (like cobalt-chromium alloys or titanium alloys), polymers (like carbon fibers), and biologically inert ceramics (like aluminum oxide or silicon carbide). These materials shared the characteristic of high mechanical strength but required a second surgery for removal. With the deepening of medical biomaterials research, both natural and synthetic biodegradable polymeric materials have garnered increased attention. Representing biodegradable polymeric materials, hydrogels have significant advantages in their ability to load osteoinductive molecules, drugs, growth factors, and nanoparticles. Through precise control of hydrogel synthesis and modification, the release profiles of these critical components and the mechanical characteristics of the hydrogel can be finely tuned, making hydrogels an ideal choice for designing bone repair materials [16].

1.2. Inherent properties of chitosan

Chitin is one of the largest natural macromolecules on earth, found extensively in the shells of shrimp and crabs, the exoskeletons of various insects, as well as the cell walls of mushrooms and fungi [17]. Chitosan (CS), as depicted in Fig. 1, is a natural macromolecule compound derived from the deacetylation of chitin. In many previous studies, chitosan has been shown to be biocompatible, biodegradable, and low-immunogenic in a variety of systems [18], and has significant biological activities, including scavenging of free radicals, chelating of metal ions, modulation of antioxidant enzymes, and reduction of lipid peroxidation, among other antioxidants [19]. It also exhibits anti-tumor, anti-inflammatory, antimicrobial, immunomodulatory, anti-obesity, and various other effects [20,21]. Moreover, CS plays a pivotal role in guiding bone regeneration, increasing bone density, and promoting fracture healing [22]. Given these remarkable attributes, CS is considered as an ideal bone repair material.

1.3. Chitosan-based hydrogel in bone defects

Significant advances have been made in the field of chitosan-based hydrogels for bone repair in recent years. This includes the development of stimuli-responsive hydrogels, microsphere hydrogels, nanohydrogels, and metal ion hydrogels. These innovative applications continue to produce exciting new results. For example, microsphere hydrogels provide precise localized drug delivery to improve therapeutic efficacy [23]. Nanohydrogels take advantage of nanotechnology to enhance drug release kinetics and tissue integration, while metal ion hydrogels utilize the potential of metal ions to enhance regenerative properties. Organized in these three forms is the functional form of the hydrogel, often embodied in the stimulus response, with stimulus-responsive hydrogels adjusting their properties in response to environmental cues, making them ideal for dynamic and customized therapies [24]. These advances not only expand the toolbox for bone repair but also enrich the potential of chitosan hydrogels in this critical area.

In this review, chitosan-based hydrogels are classified into three categories according to the type of tissues they are used for in bone repair: chitosan-based nanohydrogels, chitosan-based hydrogel microspheres, and chitosan-based metallic gels. The special properties of these hydrogels are further summarized, including multi-responsive characteristics such as pH-responsive, temperature-responsive, and bioactive-responsive (Table 1). This review aims to provide a comprehensive overview of the applications of different types of CS-based hydrogels in bone repair-related materials. By amalgamating the current state of knowledge and recent advancements, it intends to offer fresh and innovative ideas for researchers to develop novel CS-based bone repair materials and promote the potential applications of CS-based hydrogels (Fig. 2).

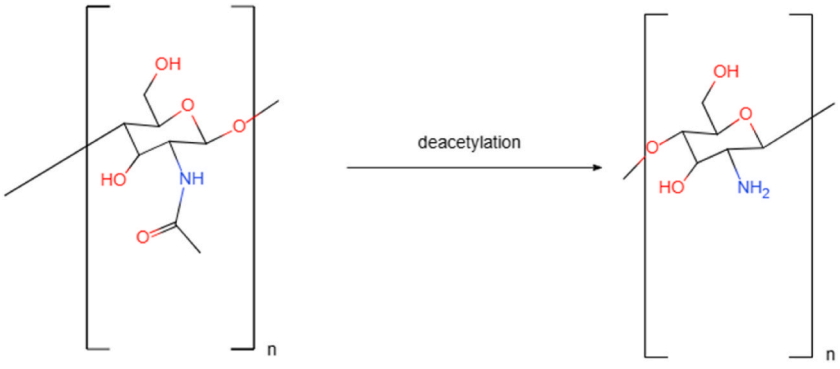

Fig. 1. Source of chitosan, with deacetylation of chitin.

Table 1
Main forms of chitosan-based hydrogels.

Types	Components	Preparation method	properties	Reference
Metal-ion doping	Metal-Organic Frameworks, Carboxymethyl Chitosan, Dextran, 4-formylphenylboronic acid	Double-network	pH/Bioactive responsive	[25]
	Acrylamide, Mo2Ti2C3, Deacetylated chitosan, Gelatin	Chemical crosslinking	Bioactive responsive	[26]
	Poly (acrylamide), Chitosan	Double-network	Bioactive responsive	[27]
Microsphere doping	Quercetin loaded-Zein microsphere, Chitosan, Basil seed gum	Physical crosslinking	Antioxidant (Bioactive responsive)	[28]
	Chitosan microsphere, Hydroxypropyl chitin	Physical crosslinking	Temperature responsive	[29]
	Bone ECM/Oleoyl chitosan, Gelatin microsphere	Physical crosslinking	Bioactive responsive	[30]
	Quaternized chitosan, PF127-CHO/	Chemical crosslinking	pH responsive	[31]
	Alginate microsphere, Chitosan/Glycerophosphate	Physical crosslinking	Bioactive/Temperature responsive	[32]
Nanoparticle doping	MgFe-layered double hydroxide nanosheets, Chitosan/silk fibroin	Physical crosslinking	Temperature responsive	[33]
	Cellulose nanofibers/nanocrystal, chitosan-beta-glycerophosphate salt	Physical crosslinking	Temperature/pH responsive	[34]
	Chitosan Methacrylate, Graphene Oxide	Chemical crosslinking	Photo Responsive	[35]
	Methylpropenylated hyaluronic acid, Chitosan oligosaccharide, mesoporous silica nanoparticles	Double-network	Temperature responsive	[36]

2. Preparation and modification strategies of CS-based hydrogels

2.1. Crosslinking methods for CS-based hydrogels

Hydrogels can be categorized into various types based on their crosslinking methods, morphology, gel properties, mechanical capacity, functional performance or responsive behavior. There are several processes available for preparing CS-based hydrogels, including chemical crosslinking, physical crosslinking, enzymatic crosslinking, ultraviolet (UV) crosslinking, gamma irradiation [37] and electron beam crosslinking [38,39]. When tailored for applications in bone repair, the crosslinking processes for CS-based

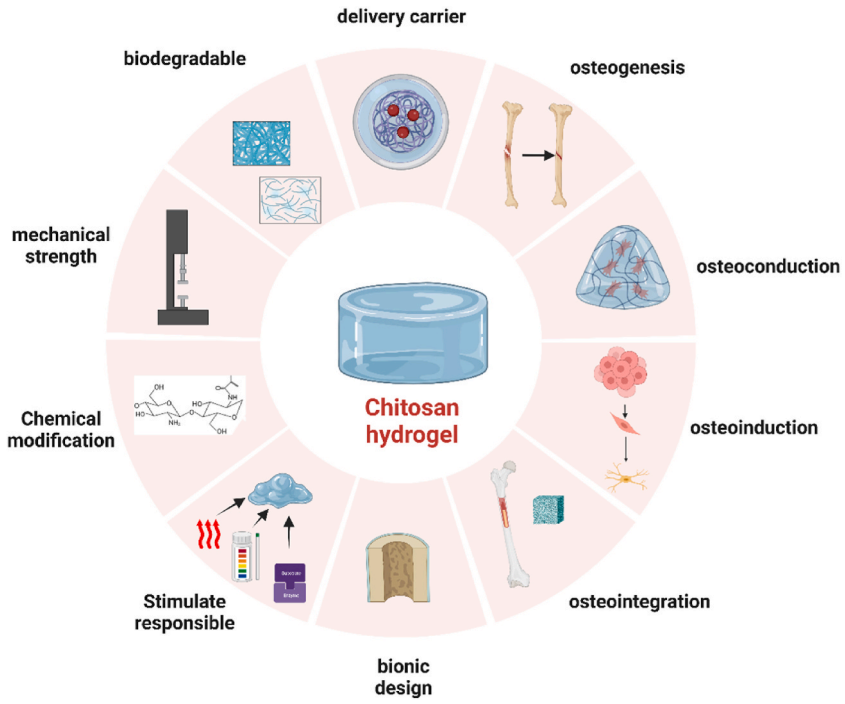

Fig. 2. Preparation of CS-based hydrogels and their role in osteogenesis (publication license from biorender).

hydrogels predominantly fit into two principal categories: chemical crosslinking and physical crosslinking. In physical-crosslinked hydrogels, the chemical chains of the polymer are always held together by molecular entanglement or non-covalent bonding formed by secondary interactions (mainly including hydrogen bonding) [40], ionic interactions [41] and hydrophobic interactions [42]. The key characteristic of physical-crosslinked hydrogels is their reversibility, attributed to these bonds, rendering them amenable to self-healing properties. This quality proves advantageous in the realm of CS hydrogels for medical applications [43]. Generally, physical-crosslinked hydrogels formed through physical crosslinking exhibit good biocompatibility without the addition of any crosslinking agents. Nevertheless, some drawbacks, such as poor mechanical performance and difficulty in controlling their average pore size, are inevitable in such physical crosslinked hydrogel systems.

Comparatively, chemical-crosslinked hydrogels entail the binding of polymer chains through irreversible covalent bonds. These chemical-crosslinked hydrogels typically exhibit the enhanced mechanical robustness and stability owing to the presence of stronger chemical linkages. Nonetheless, a noteworthy concern arises from certain crosslinking agents in chemical crosslinking processes, such as glutaraldehyde, which are known to exhibit biotoxicity, thereby compromising the hydrogel's biocompatibility [44,45]. Genipin, a natural crosslinking agent extracted from the gardenia plant [46], has significantly lower cytotoxicity compared to glutaraldehyde and higher excellent antibacterial properties, reducing the risk of bacterial attachment after scaffold implantation [47]. There is an ongoing exploration of safer and less toxic chemical crosslinking agents, exemplified by substances like tripolyphosphate [48] and vanillin [49], in the formulation of hydrogels. Therefore, the search for safe, non-toxic and cost-effective chemical crosslinking agents is a promising direction for advancing the utilization of hydrogels in the biomedical domain, particularly in the context of bone repair.

Double-network (DN) hydrogels, a subset of physically crosslinked hydrogels, have emerged as compelling candidates in clinical research for potential use as bone repair materials. Their appeal lies in their remarkable load-bearing capacity and minimal friction characteristics [50]. DN hydrogels feature a unique structure, comprising two asymmetric networks, each possessing distinct qualities (Fig. 3), resulting in a material that is both rigid and flexible [51]. The rigid network serves as a sacrificial binder to effectively disperse energy, while the flexible network maintains structural integrity during deformation [52,53]. This principle is considered to be broadly applicable in a variety of rigid materials, including polymers, metals, and ceramics [54]. Bi et al. have developed surface-mineralized physically crosslinked PVA/CS DN hydrogels [55]. In a low-temperature environment, PVA molecular chains can form crystalline regions and interact with CS chains to establish stable hydrogen bonds, thereby enhancing their thermal stability. However, manufacturing conductive hydrogels with high stretchability, excellent toughness, outstanding sensitivity, and low-temperature stability is still a significant challenge. These hydrogels are biocompatible and non-hemolytic, making them suitable for applications in bone repair.

CS has a significant number of active amino and hydroxyl groups, which allows to form various types of CS-based hydrogels through chemical and physical crosslinking. On this basis, CS-based hydrogels can be further subdivided into chitosan-based nanohydrogels, chitosan-based hydrogel microspheres, and chitosan-based metallic gels.

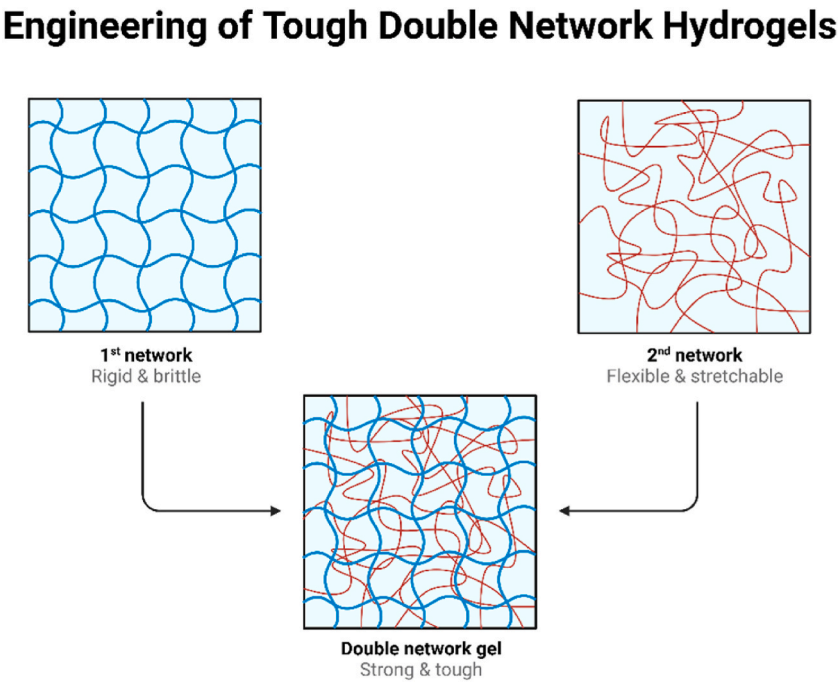

Fig. 3. Dual-network crosslinked hydrogel composition forms.

2.2. CS modification techniques

Another direction in developing CS-based hydrogels for bone repair involves the use of chitosan derivatives, namely modified chitosan. Recently, there has been increasing interest in structurally modifying chitosan to enhance the solubility of these CS derivatives and expand their application in bone repair [46]. One study has shown that chitosan with introduced substituents can reduce intracellular reactive oxygen species (ROS), thereby promoting osteogenic differentiation of mesenchymal stem cells (MSCs) [56]. Therefore, chitosan modification techniques can selectively enhance the biological activity of chitosan and further influence the biological activity of CS-based hydrogels. Presently, chitosan modification techniques encompass a spectrum of derivatives, including anionic chitosan derivatives like carboxymethyl chitosan, celebrated for its improved solubility, calcium ion adsorption capacity, and degradability [57]. Strong cationic water-soluble chitosan derivatives such as hydroxypropyl trimethylammonium chloride and guanidinium chitosan are also in the spotlight [58]. Sulfated chitosan has shown promise in augmenting osteoinduction by enhancing BMP-2 bioactivity [59]. Furthermore, glycol chitosan, known for its solubility across alkaline, neutral, and acidic conditions, presents yet another dimension of versatility [60]. These diversified chitosan derivatives yield a rich repository of raw materials, significantly enhancing the potential applications of CS-based hydrogels in the domain of bone repair.

3. Stimuli-responsive CS-based hydrogel

Chitosan, serving as the primary component, offers a versatile platform for the creation of stimulus-responsive hydrogels. These hydrogels can be further customized by combining chitosan with other polymers through various cross-linking methods, including photopolymerization, thermal polymerization, and polymer-polymer cross-linking. Recent studies have highlighted the significance of injectable in-situ molding hydrogels in orthopedic applications [61]. In these applications, bone regeneration at the defect site depends primarily on the physical gelation of CS-based injectable hydrogels. These hydrogels act as short-lived scaffolds that gradually degrade and stimulate the proliferation and differentiation of osteoblasts, ultimately promoting bone repair [62]. Among the wide variety of hydrogels available, those have sensitivity sensitive to temperature and pH have proven to be very effective in the management of major bone defects. The significant advantage of these hydrogels is their ability to be injected in hard-to-reach anatomical sites and to mold the gel state to the shape and size of the injury. Notably, these hydrogels do not depend on the use of organic solvents, co-polymerizing agents, or external triggers to initiate gel formation [63].

On the other hand, stimulus-responsive hydrogels span several categories contingent upon the type of stimulus they react to. These categories encompass physical-responsive stimuli (e.g., temperature, pressure, light, electricity, and magnetism), chemical-responsive stimuli (e.g., pH, ionic strength, and glucose), and biologically-responsive stimuli (e.g., DNA, enzymes, and antigens). In the mainstream of CS-based hydrogels, three prominent types stand out: temperature-responsive, pH-responsive hydrogels and ROS-responsive hydrogels. These versatile hydrogels are primed to offer innovative solutions in various biomedical applications.

3.1. Temperature-sensitive CS-based hydrogels scaffold

Thermosensitive hydrogels are a class of hydrogels that respond to changes in temperature. These hydrogels exhibit a fluid-like behavior below the critical solution temperature (CST) and undergo a transition from a solution state to a gel state above the CST. Thermosensitive hydrogels with critical solution temperatures close to physiological conditions hold significant promise as biomaterials for applications in bone tissue engineering [64]. CS, in its natural form, lacks inherent thermo-sensitivity. To confer thermosensitive properties, it necessitates the incorporation of specific hydroxyl groups or amphiphilic thermosensitive polymers into the chitosan network [65]. This section highlights a series of CS-based thermosensitive hydrogels that have been reported for the treatment of bone injury and bone tissue regeneration.

Chitosan/β-glycerophosphate (CS/GP) hydrogels have received much attention for their potential to efficiently deliver a variety of biologically active molecules, drugs, and growth factors, as well as to provide structural support for the development of complex multilayer systems involving cells and tissues [66]. Thermo-gelation of these hydrogels was achieved by introducing glycerophosphate into chitosan solution. On this basis, researchers have made significant progress in fabricating injectable thermostable hydrogels using the CS/GP system. For example, they enhanced the performance of the system by incorporating negatively charged acid-soluble collagen (ASC) to mimic a bone-like environment [67]. In addition, to address the lack of osseointegration and poor mechanical strength of this type of hydrogel, researchers introduced materials like tri-calcium phosphate (TCP), nanohydroxyapatite (nHAp), nano-bio-glass-ceramics (nBGC), and wollastonite (WS). These additions have successfully bolstered bone-forming capabilities [68]. However, despite the continuous improvement of this class of hydrogel through polymer additives, bioceramic encapsulation, drug delivery, and cellular loading, there is still no CS/GP combination capable of simultaneously displaying the biological and mechanical properties required for ideal bone tissue engineering [66].

Poly (N-isopropylacrylamide)-based hydrogels: PNIPAM or PNIPAAm is a temperature-responsive polymer that is widely used as a thermo-responsive unit in thermosensitive CS-based hydrogels. Cross-linking chitosan hydrogels with PNIPAM increases their adhesion to cells, which improves mechanical properties and cell proliferation [69]. In the realm of innovative hydrogel systems, the combination of allyl-modified chitosan (OAL-CS) with thermo-responsive PNIPAM results in the rapid formation of a cross-linked hydrogel network through "thiol-alkene" click chemistry. Notably, this hydrogel demonstrates a non-toxic profile once exposure to human bone marrow mesenchymal stem cells. Furthermore, histological assessments following subcutaneous injection for 5 days reveal no signs of inflammation in the surrounding tissues [70]. In another injectable thermosensitive system based on hyaluronic acid (HA) and PNIPAM, HA-CS-PNIPAM is combined with biphasic calcium phosphate (BCP) to create a cell delivery system that induces

osteogenesis. The application of HA-CS/PNIPAM/BCP effectively enhances the proliferation rate and alkaline phosphatase (ALP) activity of human fetal osteoblasts. This combination also leads to increased expression of vital genes in osteoblasts, enhanced extracellular matrix mineralization, elevated calcium accumulation, and an overall boost in mechanical strength. Additionally, subcutaneous injection of hydrogels containing cells for 3 months results in the formation of ectopic bone tissue in nude mice [71]. The versatility of CS/PNIPAM-based scaffolds extends to their capacity for incorporating drugs, cells, growth factors, or genes to stimulate the effective repair of bone defects [72]. For example, the researchers utilized ultrasound to fabricate chitosan-grafted PNIPAM (CS-g-pN). Curcumin was then added to the CS-g-pN nanogels using an incubation method, resulting in the successful development of curcumin-containing nanogels. The cytotoxicity evaluation confirmed that the loaded drug maintained better biocompatibility [73]. This illustrates the tremendous potential of CS/PNIPAM-based hydrogels in the realm of bone tissue engineering and regenerative medicine.

Thus, thanks to the properties of temperature-sensitive hydrogels, several points should be kept in mind when designing CS-based thermosensitive hydrogels for biomedical applications, such as the selection of suitable temperature-sensitive polymers based on the route of administration, the adjustment of the porosity of the hydrogel to control the drug release, the mechanical properties, and the time required for sol-to-gel formation [74]. These thoughtful considerations are paramount for the successful development and utilization of CS-based thermosensitive hydrogels in the field of biomedicine.

3.2. *Application of pH-responsive hydrogels scaffold in bone repair*

pH-sensitive hydrogels are synthetic polymers that respond to different pH values. When the pH of the microenvironment changes, hydrogels alter their mechanical properties through a protonation/deprotonation mechanism. Therefore, the alternation of the chemical structure of polymer hydrogels can be controlled by pH [75]. pH-sensitive hydrogels are typically categorized into three primary classes: anion-sensitive, cation-sensitive, and amphoteric ion-sensitive hydrogels. CS-based hydrogels, featuring multiple amino groups, can be tailored to form cationic pH-responsive hydrogels [76]. For bone repair, this cationic responsiveness equips hydrogels to release drugs within an acidic microenvironment effectively. This strategy aligns with the treatment of infected bone defects, where the microenvironment often exhibits an acidic profile [77]. A notable example is asymmetric microfluidic/chitosan device developed by Chen et al. for pH-responsive drug release without the need for external stimulation. This innovative system boasts biocompatibility and selective drug release properties. Additionally, in vitro antimicrobial tests have affirmed the hydrogel's excellence in pH-responsive antimicrobial drug release [78]. Conversely, hydrogel responding to alkaline environments is relatively less common, primarily because bone defect site tends to exhibit acidity in cases of infection or a neutral pH environment. As a result, most researchers concentrate their efforts on the design of CS-based hydrogels that are specifically sensitive to neutral pH environments. This strategic focus aligns with the predominant pH characteristics observed in the context of bone tissue repair and regeneration [79]. For instance, researchers have devised a composite hydrogel by combining carboxymethyl chitosan (CMCh) with amorphous calcium phosphate (ACP), which is capable of self-assembly at pH 11. However, when doped with glucono δ-lactone acidifier, the self-assembly pH is reduced to 7.5. This transformation results in pH-responsive injectable hydrogel with remarkable biocompatibility that promotes effective cell adhesion and proliferation. Furthermore, it enhances the expression of bone markers and promotes the regeneration of new bone [80].

The Schiff base reaction plays a pivotal role in the formation of pH-responsive hydrogels. Schiff base bonds typically arise in neutral or mildly acidic conditions, but they may undergo hydrolysis when exposed to alkaline conditions [81]. This inherent behavior renders Schiff base bond-containing hydrogels stable under neutral pH environments while bestowing upon them a distinctive pH-responsive quality [82]. As an example, Pan et al. ingeniously combined the -CH=O functional group of oxidized sodium alginate (OSA) with the $-NH_2$ functional group of glycol chitosan (GCS), incorporating calcium phosphate nanoparticles (CaP NPs) to create reversible Schiff base linkages. This innovative approach allowed for the on-demand fabrication of a novel, biocompatible injectable self-repairing nanohybrid hydrogel. Notably, this hydrogel exhibited favorable pH-responsive swelling behavior and biodegradability. Moreover, it demonstrated a remarkable ability to stimulate the proliferation, differentiation, and osteoinduction of bone marrow mesenchymal

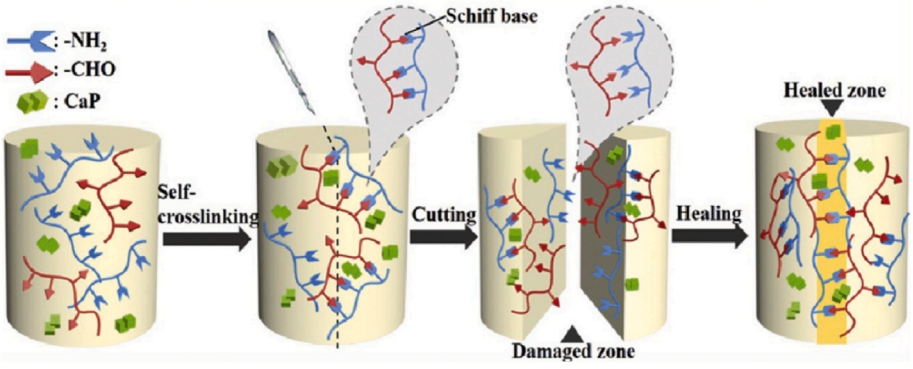

Fig. 4. pH-responsive hydrogels based on reversible Schiff bases, Reproduced from Ref. [83] with permission from Copyright 2024 Elsevier.

stem cells in vitro [83] (Fig. 4). These innovations underscore the significant potential of pH-responsive CS-based hydrogels in biomedical applications, particularly in the context of bone tissue engineering.

3.3. Biologically stimuli-responsive hydrogels scaffold in bone repair

Stimulus-responsive materials for bone defect repair have recently garnered significant attention due to the distinct environmental conditions surrounding injured areas, such as variations in reactive oxygen species (ROS) concentration, enzyme concentration, and other factors, compared to healthy tissues. Among the various biomaterials capable of responding to these biologically based stimuli, chitosan-based hydrogels stand out. These biocompatible polymers with specific functional groups provide a favorable platform for modulating the local microenvironment. Their solubility, hydrophilicity, biodegradability, and chemical behavior can be easily modified according to bioenvironmental conditions. The functional groups of chitosan (amines and hydroxyls) are physically or chemically linked to ROS [84], GSH [85], enzymes [86], or other components to design stimuli-responsive hydrogels.

In bone-related microenvironments, especially post-fracture scenarios, studies on biostimuli-responsive chitosan-based hydrogels have primarily focused on ROS responsiveness. Increased ROS production in the local microenvironment after a fracture significantly affects the healing process [87]. The ROS responsiveness of chitosan is mainly reflected in two aspects. Firstly, chitosan can activate antioxidant pathways in human cells to prevent oxidative stress generated by ROS in diseased areas [88]. Secondly, chitosan has inherent antioxidant activity as a ROS scavenger, which prevents peroxidation and degradation of cell membranes, lipids, proteins, and genomes [89]. This unique property results from the interaction of free radicals (ROS) with the reactive amino and hydroxyl groups of chitosan, stabilizing the free radicals. However, in the field of bone defect repair, researchers have paid little attention on the direct use of ROS-responsive chitosan to make chitosan-based hydrogel scaffolds [90,91]. More researches have focused on utilizing aggregates of multiple ROS-responsive substances to respond to the local oxidative microenvironment for the delivery of drugs or other bioactive factors [92]. This may be related to the microscopic scale of ROS scavenging, with chitosan-based hydrogels made in the form of nanometer or microspheres offering a more appropriate microscopic scale to provide a larger specific surface area for reaction areas.

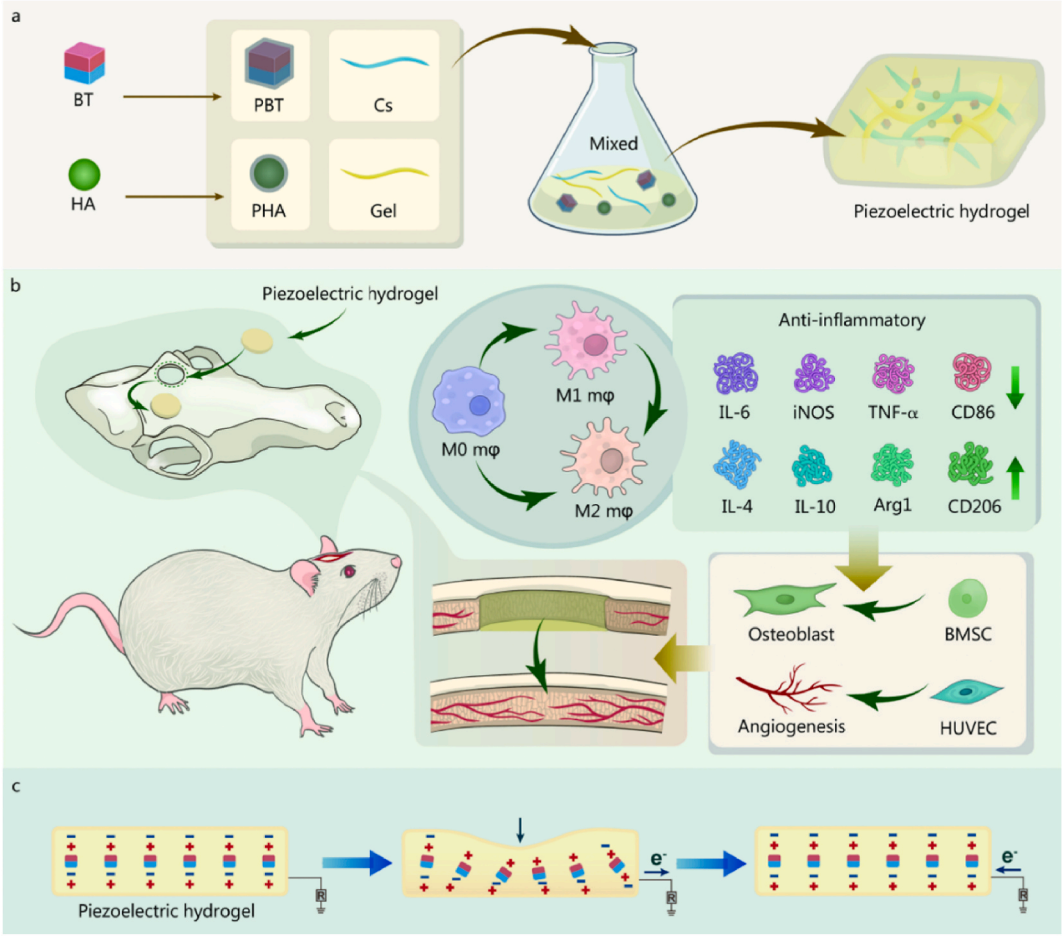

Fig. 5. Schematic illustration of the design strategy of piezoelectric CG/PHA/PBT hydrogels and the bone regeneration mechanism (Reproduced from with [99], permission from Creative Commons CC BY license).

4. CS-based nanomaterials hydrogels

Nanogels, typically formed through chemical or physical cross-linking, maintain their nanostructure under physiological conditions. Nanogels can serve as excellent carriers for drugs and macromolecular bioactive agents that can be incorporated into the hydrophilic network of the nanogel while maintaining the bioactivity of enzymes or the conformation of proteins. In addition, nanogels allow for precise control of drug release and are hybrid carriers, combining the advantages of hydrogels and nanoparticles [93,94]. When it comes to hydrogels designed for bone repair, the introduction of functional nanoparticles (NPs) can significantly enhance their mechanical, biological, and chemical properties, thereby broadening their potential applications. Notably, a variety of nanoparticles, including metal/metal oxide nanoparticles (such as gold, magnesium oxide, and iron tetraoxide), inorganic/ceramic nanoparticles (e.g., hydroxyapatite, calcium phosphate, silica, silicate, and graphene oxide), and polymer nanoparticles (comprising natural/synthetic polymers, dendritic polymers, and hyperbranched polyesters), can effectively promote osteogenic differentiation and mineralization for bone regeneration [15,95–97].

A representative direction is to modify the physical properties of the hydrogel itself by adding nanoparticles, for example, nano-hydroxyapatite modifies the hydrogel network by promoting the secondary hydrogen bonds thereby enhances the mechanical stiffness [68]. Another interesting experiment also revealed the role of beta-tricalcium phosphate on the solution-gel phase transition of chitosan-based hydrogels, which reflects the interaction between nanoparticles and hydrogels rather than utilizing hydrogels as carriers alone [98]. Moreover, emerging piezoelectric sensing nanoparticles are gaining more attention, this class of nanoparticles utilizes the conductive properties of chitosan to make bioelectrical stimulation-responsive bone regeneration possible. In a study, researchers incorporated PDA-modified barium titanate (PDA-$BaTiO_3$, PBT) nanoparticles into a chitosan/gelatin (Cs/Gel) matrix to create porous piezoelectric hydrogel bone scaffolds (Fig. 5). These scaffolds harnessed their endogenous piezoelectric stimulation and bioactive components, demonstrating cytocompatibility, along with immunomodulatory, angiogenic, and osteogenic capabilities [99].

In the context of infectious bone repair, nanogels have also garnered increased interest due to their exceptional drug-carrying and slow-release capabilities. In one study, spray-dried PLGA/PLA nanoparticles loaded with linezolid were introduced into CS-based hydrogels prepared using a nano-spray-drying technology. This innovation effectively mitigated bone infections, leading to positive clinical, biochemical, radiological, and histopathologic outcomes [100]. Furthermore, nanogels also have the advantage of responding to external stimuli. Stimulus-responsive nanogels are widely used in delivery systems, especially in the field of tumor-targeted delivery and controlled release, for example, in response to pH or glutathione (GSH) [101]. Therefore, their application in the context of bone tissue repair or bone tumor therapy is still a burgeoning field with immense promise, paving the way for further research and exploration.

5. CS-based hydrogel microspheres

In bone regeneration, microsphere structures help to reduce side effects and immune rejection of implants and scaffolds. To prepare hydrogel scaffolds containing microspheres, the prepared microspheres can be homogeneously dispersed in a hydrogel precursor solution [102] or a monomer solution [103], and then, depending on the mode of hydrogelation, the hydrogel containing microspheres can be obtained by physical cross-linking or chemical cross-linking to obtain hydrogels containing microspheres. Among them, physical cross-linking mainly including thermal cross-linking [104], ionic gel cross-linking, electrostatic interaction cross-linking [105], etc., and chemical cross-linking mainly includes enzymatically dissociated cross-linking [106], Schiff base cross-linking [107], Michael addition cross-linking [108], etc. With their unique porous structure and good biocompatibility, hydrogel microspheres not only play a unique role in maintaining cell viability, but also, similar to nanoparticles, promote the long-term release of growth factor targeting thanks to the combination of microspheres and hydrogel [109]. Compared to nanoparticle hydrogels, microsphere hydrogels are distinguished by their freer size, which facilitates the carrying of bioactive macromolecules. A noteworthy example is the work of Ji Li et al., who engineered titanium alloy (Ti6Al4V) scaffolds with the inclusion of rhBMP-2 (recombinant human bone morphogenetic protein-2) microspheres and a heat-sensitive chitosan Thioglycolic acid (CS-TA) hydrogel. This composite scaffold, featuring porosity, thermo-sensitivity, and mechanical strength, was designed to provide sustained release of rhBMP-2, significantly enhancing biocompatibility and osteogenic potential [110]. Furthermore, the incorporation of near-infrared (NIR) light-responsive polydopamine-encapsulated calcium magnesium carbonate microspheres in a thermo-responsive hydroxybutyl chitosan hydrogel, as developed by Wan et al., achieved the sequential delivery of both aspirin and osteogenic bone morphogenetic protein 2 (BMP-2). This approach was successfully validated using a mouse bone defect model, demonstrating its efficacy in promoting bone healing [111].

These studies highlight the vital role of microsphere-hydrogel composites as platforms with slow-release capabilities and inherent bioactivity. Through the delivery of growth factors, drugs, and others, it is evident that such microsphere-hydrogel combinations are poised to become integral components of future strategies in the field of bone repair [112]. This innovative approach holds immense potential for enhancing the outcomes of bone regeneration therapies.

6. Metal ion cross-linked CS-based hydrogels

With advancements in bioactive materials, the incorporation of metal ions into biomaterials has emerged as a powerful strategy for stimulating osteoblast activity, promoting angiogenesis, inhibiting osteoclast and bacterial activity, and thereby finding vital applications in bone defect repair [113–115]. Various metal ions, including Cu^{2+}, Mg^{2+}, Sr^{2+}, gallium ions (Ga^{3+}), and Co^{2+}, have been

identified as contributors to improving the microenvironment of bone defects and facilitating bone repair [116–118]. Integrating these metal ions into hydrogel materials, known for their injectability and plasticity, proves to be a valuable loading strategy. Such incorporation can be achieved through physical mixing or chemical bonding [114], ultimately enhancing the hydrogel's strength, which is essential for preserving its structure and preventing excessive degradation [119].

When it comes to CS-based hydrogels, their weak amino groups often necessitate indirect cross-linking through intermediates. As mentioned above, previous research frequently involved the direct doping of metal or its oxide nanoparticles to create metal nanoparticle-CS-based hydrogels [120]. However, the ability of chitosan to form stable chelates with therapeutic metal ions has recently received much attention. Coordination bonds between metal ions and chitosan functional groups (amino and hydroxyl groups) can improve the delivery of therapeutic metal ions and thus enhance the biological activity of the polymer [121]. Andrea Lončarević et al. conducted an in-depth study on the effect of coordination between chitosan-based hydrogels and single and bimetallic ions on the mechanical properties of the gels, and found that the addition of metal ions significantly enhanced the hydrogels' modulus of energy storage and loss as well as the overall enhancement of their structural strength [122,123]. This provides a good direction for the further use of chitosan-based metal gels for bone defect repair. A notable example comes from Xiong et al. who exploited the ability of Mg^{2+} to form ligand-bonded crosslinks with BP (Fig. 6) to develop covalently bonded crosslinked hydrogels using polyacrylamide (PAM), chitosan, and bisphosphonates (BP). These chitosan-BP/PAM/Mg^{2+}-BMP hydrogels promoted bone tissue growth and treatment of bone defects through localized releasing of Mg^{2+} and BMP [27]. Characterization analysis showed that Mg^{2+} altered the pore distribution of the hydrogels from a disordered to a uniform distribution and prevented thermal decomposition. In another instance, Lu et al. devised a bone repair scaffold utilizing sodium alginate and carboxymethyl chitosan to enable sustained Cu^{2+} release. Cu^{2+} demonstrated no significant impact on the biosafety of MC3T3-E1 cells in cell adhesion and proliferation assays. Cytoskeletal staining and alizarin red staining illustrated that Cu^{2+} promoted cell adhesion, osteogenesis and increased osteogenic gene expression, bone mineralization, and angiogenesis. Furthermore, the release of Cu^{2+} bolstered the scaffold's antimicrobial properties [124].

Importantly, the application of metal ion crosslinked chitosan hydrogels extends beyond bone defect repair alone. Several studies have showcased their remarkable efficacy in treating bone defects in osteoporotic patients, high-glucose environments, and infected conditions [125]. This versatility underscores the potential of metal-ion-loaded chitosan hydrogels in diverse clinical scenarios, emphasizing their multifaceted benefits in bone regeneration.

7. Conclusion and outlook

This review provides a comprehensive overview of the recent advancements in CS-based hydrogels for bone repair. Through a systematic review, it offers insights into the preparation methods of CS-based hydrogels, highlighting the latest developments across various hydrogel types and addressing their respective limitations. These insights collectively underscore the promising prospects for the utilization of this biocompatible and degradable material in the context of bone repair. To begin with, CS-based hydrogels, crafted via diverse cross-linking techniques, offer the advantage of tunable mechanical and physical properties. Their impressive mechanical strength, coupled with a three-dimensional structure, closely resembles the microenvironment of natural bone tissues. This mimicry not only provides essential support and guidance for osteoblasts but also fosters bone regeneration. Moreover, the pH sensitivity and compatibility with nanoparticles and microspheres render CS-based hydrogels highly proficient in controlled drug release. This capability enables the gradual release of growth factors, medications, or bioactive molecules, thereby expediting the bone healing

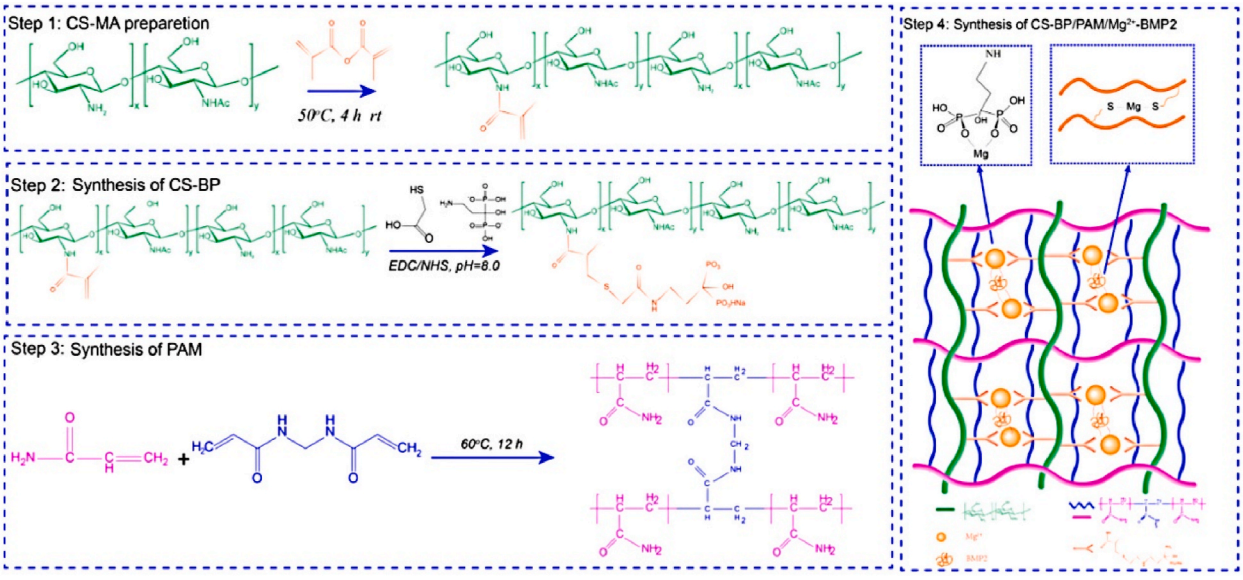

Fig. 6. Preparation of metal ion cross-linked hydrogels Reproduced from Ref. [27]with permission from Copyright 2024 Elsevier.

process. Furthermore, the adaptability of heat-sensitive and injectable CS-based hydrogels, characterized by their plasticity and customizability, makes CS-based hydrogels suitable for a wide spectrum of bone injuries and repair needs, ranging from minor fractures to extensive bone defects. Additionally, the incorporation of metal-ion cross-linking in chitosan augments the mechanical properties of these hydrogels while concurrently promoting bone repair. Consequently, the application of CS-based hydrogels in bone repair offers an innovative, effective, and sustainable approach to fracture healing and addressing bone defects. These hydrogels hold the potential to be an important tool in the future of orthopedics.

In addition to the excellent properties mentioned above, CS-based hydrogels also face several problems. Firstly, even after substantial reinforcement and modification, their mechanical properties still fall short of the strength exhibited by metal scaffolds. This limitation may restrict their application in load-bearing scenarios, particularly when addressing extensive critical bone defects. Secondly, the inherent biodegradability of CS-based hydrogels can lead to a decline in sustained performance during the repair process. To overcome this issue, researchers will need to meticulously control and fine-tune the balance between providing adequate support and maintaining stability in these hydrogels. Furthermore, for clinical applications, the preparation and application of these hydrogels demand specialized techniques and equipments. This factor can potentially increase the complexity and cost of treatment. In summary, CS-based hydrogels represent a promising solution for addressing bone defects, but it's crucial to acknowledge and work on these challenges to fully harness their potential in the field of bone repair.

Statements

During the preparation of this work the author used ChatGPT in order to improve readability and language of the work. After using this tool, the author reviewed and edited the content as needed and take full responsibility for the content of the publication.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Jianyang Shan: Writing – review & editing, Writing – original draft. **Yaling Yu:** Validation, Supervision. **Xiaohan Liu:** Visualization. **Yimin Chai:** Supervision, Funding acquisition. **Xing Wang:** Validation, Supervision. **Gen Wen:** Supervision, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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