Review

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Hyaluronic acid as a bioactive component for bone tissue regeneration: Fabrication, modification, properties, and biological functions

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Abstract: Hyaluronic acid (HA) is widely distributed in the human body, and it is heavily involved in many physiological functions such as tissue hydration, wound repair, and cell migration. In recent years, HA and its derivatives have been widely used as advanced bioactive polymers for bone regeneration. Many medical products containing HA have been developed because this natural polymer has been proven to be nontoxic, noninflammatory, biodegradable, and biocompatible. Moreover, HA-based composite scaffolds have shown good potential for promoting osteogenesis and mineralization. Recently, many HA-based biomaterials have been fabricated for bone regeneration by combining with electrospinning and 3D printing technology. In this review, the polymer structures, processing, properties, and applications in bone tissue engineering are summarized. The challenges and prospects of HA polymers are also discussed.

Keywords: bioactive component, hyaluronic acid, bone regeneration, 3D printing, tissue engineering

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1 Introduction

Hyaluronic acid (HA), also known as hyaluronan or hyaluronate, exists in all body fluids and the extracellular matrix (ECM) of many tissues [1,2]. It is responsible for regulating tissue hydration and adjusting the viscoelasticity of some body fluids, such as synovial fluid in the joint cavity [3]. It also regulates fundamental cellular processes, including cell adhesion, proliferation, and differentiation, by binding with surface receptors of target cells [4,5]. In addition, HA participates in many biological responses, including angiogenesis, inflammation, wound healing, and bone regeneration [6,7]. HA-based medical productions have been in clinical use in drug delivery, cosmetics, and tissue regeneration for over 40 years because of their outstanding biodegradability and biocompatibility properties [8,9].

At present, the repair of large bone defects due to infection, trauma, surgical resection of tumor, and malformation still remains a major challenge in modern medicine [8,9]. The gold standard for the treatment of large bone defects is bone transplantation, including autografting and allografting [10–12]. However, many problems arise with this treatment approach, such as limited sources, immunological rejection, infection, donor-site morbidity, and graft resorption [13,14]. Bone substitutes fabricated by the tissue-engineered methods are rapidly becoming promising alternatives for the treatment of bone defects [15,16]. In recent years, given the increasing demand for the repair and replacement of bone tissue, various tissue-engineered bone scaffolds loaded with or without cells have been fabricated for bone regeneration. Owing to its favorable biochemical properties, HA, as well as other common natural polymers such as alginate,

collagen, gelatin, silk fibroin, fibrin, and chitosan, has already been utilized to construct various tissue-engineered bone scaffolds in the field of bone repair with a rich research history [17]. As a natural component of ECM, HA can not only provide osteogenesis-related cells with a similar extracellular environment but also initiate many cellular signaling pathways in bone regeneration [18,19]. Additionally, the biological function and physicochemical properties of HA can be changed by chemical modification [20]. HA and its derivatives are also commonly used to mix with other gels, resulting in a hybrid hydrogel with combined characteristics. By combining with various tissue-engineered processing techniques such as freeze drying, electrospinning, and 3D printing, bone substitutes based on HA and its derivatives offer the versatility to be modified into any shapes or sizes including conversion to porous scaffolds, nanofibers, films, nanoparticles, and microspheres, for bone tissue regeneration [21,22]. Even though HA itself has a limited osteogenic effect in the process of bone repair, HA and its derivatives can be used as vehicles for osteogenesis-related cells and factors or utilized in combination with other biomaterials such as ceramics and titanium implants for bone regeneration. Therefore, we conduct this review to discuss the current status and role of HA applied in bone tissue engineering. Furthermore, the modification of HA and processing techniques of HA-based biomaterials are also discussed.

2 Properties and biological functions of HA

2.1 Structure and properties of HA

HA is an anionic, nonsulfated, and simplest glycosaminoglycan (GAG) and composed of repeating units of D-glucuronic acid and *N*-acetylglucosamine combined by β-1,4 and β-1,3-glycosidic bonds [23]. The number of repeating units in HA can reach about 10,000 or more, resulting in a molecular weight of 4 million daltons (Da). HA can bind and retain a large amount of water molecules because it is rich in negatively charged hydroxyl groups. The chemical structure of HA is shown in Figure 1. Different from other GAGs, HA is synthesized by hyaluronan synthase enzymes (HAS1, HAS2, and HAS3) in the cell membrane, instead of the Golgi apparatus, and does not attach to a core protein [24]. HA has a high molecular weight, ranging from 10^5 Da in serum to 10^7 Da in vitreous,

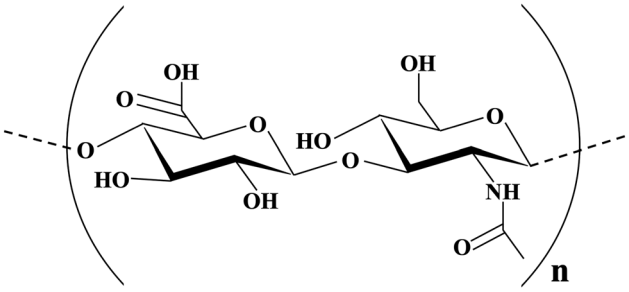

Figure 1: The chemical structure of HA. Repeating sugar molecules form the backbone of the HA chain.

and is rapidly degraded in the tissue by hyaluronidase; specifically, *N*-acetylhexosaminidase and D-glucuronidase catalyze the cleavage of glycosidic bonds to remove nonreducing terminal sugars, degrading high molecular weight HA into smaller fragments [23].

As a long-chain biopolymer in solution, HA can appear as a reinforced random coil structure with a large hydration volume, forming a stiff, viscous, gelatin-like substance by each molecule interacting with its neighbors [25]. HA is polydisperse in solution and can restrict the access of other macromolecules into its domain, which demonstrates the excluded volume effects of HA. Many current studies have demonstrated that hydrogen bonds between adjacent saccharides restrict the rotation of the glycosidic bond and contribute to the stiffening of the HA chain [26,27]. Therefore, HA forms a dynamic network, which can allow molecules with low molecular weight to penetrate freely and restrict the movements of other macromolecules with high molecular weight [25]. The solutions of HA under physiological conditions are highly viscoelastic, and the individual chains of HA remain mobile. Hence, HA can form higher order structures by interactions or combination with other proteins.

2.2 Biological functions of HA

Many studies demonstrated that HA plays an important role in cell behavior regulation, including cell proliferation, survival, motility, migration, and differentiation [28,29]. As a transmembrane receptor for HA, CD44 is highly expressed by many cells to initiate various intracellular signaling cascades. By activating CD44-mediated pathways, HA can regulate the behaviors of osteogenesis-related cells, such as cell adhesion and migration [30]. As another receptor of HA, receptor for hyaluronic acid-mediated motility (RHAMM) has multiple isoforms and can alter migratory cell behavior [31]. Additionally, the

different molecular weight and concentration of HA have different effects on cell proliferation and differentiation [29,32]. HA with low molecular weight ($<10^3$ kDa) is mostly reported to increase cell proliferation and differentiation [33]. However, the effects of HA with high molecular weight on cell proliferation and differentiation remain controversial [34,35].

HA also has bacteriostatic effect and anti-adhesive ability. Toll-like receptors (TLRs), single-pass membrane-spanning receptors, play a vital role in activating immune cell responses. HA fragments with low molecular weight can bind to TLR-4 and act as initiators for defending against bacterial infection [36]. Additionally, as a cell surface receptor of HA, ICAM-1 (intracellular adhesion molecule-1) is a member of the immunoglobulin superfamily that is expressed by lymphocytes and macrophages [37]. Currently, HA has been confirmed of its antimicrobial effects on *Aggregatibacter actinomycetemcomitans*, *b-hemolytic Streptococcus*, *Prevotella oris*, *Enterococcus*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Additionally, Drago et al. demonstrated that HA could interfere with bacterial adhesion and bacterial biofilm formation [38].

A previous study also found that HA could induce peripheral blood monocyte to express anti-inflammatory growth factors [39]. The effects of HA on anti-inflammatory functions depend on its molecular weight. HA with high molecular weight has anti-inflammatory effect, whereas low molecular weight degradation products of HA can induce various pro-inflammatory responses [40]. Additionally, HA fragments can induce macrophages to express nitric oxide, monocyte chemotactic protein-1, and macrophage inflammatory proteins [39]. Previous studies also found that HA with low molecular weight could induce the expression of inducible nitric oxide synthase (iNOS) in chondrocytes [41].

2.3 Effects of HA on bone regeneration

Bone tissue is a dense connective tissue consisting of 30% organic material and approximately 70% inorganic material. The process of bone repair includes hematoma formation, the inflammatory phase, granulation tissue formation, callus formation, and the remodeling phase [42]. Furthermore, osteogenesis-related cells, inflammatory cells, endothelial cells (ECs), and various growth factors are involved in the process of bone formation and bone remodeling. HA promotes bone regeneration by regulating cell activity and the release of biological factors. Additionally, the beneficial osteogenic effects of HA have been demonstrated by many studies. HA can bind with CD44 and be incorporated into the cytoplasm of osteoprogenitor cells to regulate the osteoprogenitor cell migration [18]. Hempel et al. found that HA could promote osteogenic differentiation of mesenchymal stem cells (MSCs) even without the existence of dexamethasone [35]. In addition, HA with different molecular weights might have different biological functions during the process of bone regeneration. Zhao et al. found that HA with low molecular weight could enhance the proliferation of bone marrow-derived mesenchymal stem cells (BMSCs), whereas HA with high molecular weight could enhance mRNA expressions of osteogenic gene markers, such as ALP, RUNX-2, and OCN [32]. Additionally, due to its good physicochemical properties, HA also can be used as a vehicle for osteogenesis-related cells and factors for bone repair.

Bone is a complex heterogeneous and vascularized tissue with vascular networks, which is connected to the blood system by transverse channels [43]. Metabolic needs and oxygen are not met when the length of bone substitutes exceeds 200 μm, resulting in core ischemia of bone substitutes and poor integration with host tissue [44]. Moreover, insufficient vascularization of bone substitutes often results in poor bone regeneration. Additionally, angiogenesis plays a vital role in the process of bone formation [45]. The fragments of HA can enhance angiogenesis via RHAMM-mediated signaling pathways in epithelial cells [46]. Additionally, HA is also responsible for promoting the proliferation and migration of ECs in the process of vessel formation [47]. Wang et al. found that HA could enhance the capillary density and blood supply during the process of tissue repair *in vivo* [48]. Ciccone et al. found that when ECs are exposed to HA, the expression of vascular endothelial growth factor (VEGF) is enhanced [49]. In another study, Matsumoto et al. utilized freeze drying technique to fabricate different sponges using HA with diverse molecular weights [50]. The *in vivo* results demonstrated that the combination of HA with low molecular weight on the sponges had a positive effect on the angiogenesis process.

3 Chemical modifications and crosslinking of HA

HA is an attractive component of artificial biomaterial fabrication in the field of tissue engineering due to its biodegradability, biocompatibility, non-immunogenicity, and non-thrombogenicity. However, native HA cannot be

a useful biomaterial for tissue regeneration owing to its poor mechanical properties and susceptibility to degradation *in vivo*. High molecular weight HA solution with high concentration is viscoelastic, and native HA molecules interact with each other by H-bonding, Van der Waals interactions, and hydrophobic forces in dilute physiological solution. However, the HA network is heterogeneous, unstable, and reversible, exhibiting weak mechanical property [23]. In fact, native HA cannot maintain its structural integrity *in vivo* and *in vitro*. Environmental factors *in vivo*, such as pH and temperature, can also change its properties [51]. HA can dissolve in aqueous solution and be degraded by hyaluronidase quickly in physiological milieu [52]. The tissue half-lives of HA are short, ranging from minutes in the blood to hours or days in skin and joints [53]. Moreover, HA is metabolized to oligosaccharides (<10 kDa) and low (4–100 kDa) or high molecular weight fragments (>100 kDa), resulting in decreased viscoelasticity of the HA solution.

In the past several decades, for better application of HA in bone regeneration, many researchers have developed various innovative technologies of hydrophobic modification to improve the properties of HA [23]. Hydrophobic modification of HA can be achieved by chemical modification of multiple functional groups [54]. Additionally, *in situ* photopolymerization can convert some kinds of liquid HA biomaterials, which contain more than one reactive group on the HA macromer, into solid constructs by exposure to light [20]. This section reviews the different methods of modified HA reported in the literature in the late years.

3.1 Chemical modifications of HA

3.1.1 Hydrazide modification

HA can be modified with adipic dihydrazide, resulting in hydrazide-modified HA. The chemical structure of hydrazide-modified HA is shown in Figure 2a. Chen et al. utilized hydrazide-modified HA to fabricate a biomimetic hydrogel system, whose physicochemical properties can be adjusted by varying the weight ratio of polysaccharides [55]. In another study, Wang et al. fabricated an injectable hydrogel by hydrazide-modified HA, which has an outstanding mechanical property to protect encapsulated human MSCs in hydrogel during the procedure of injection and maintain their osteogenic differentiation capability [56] (Figure 2b). In another study, Ossipov et al. successfully synthesized hydrazide-functionalized HA by initial mild cleavage of a disulfide bond and elimination of the generated 2-thioethoxycarbonyl moiety [57]. Additionally, hydrazide-functionalized HA can be used for continuous controlled release of osteogenesis-related factors during the process of bone repair [58]. Hydrazide-functionalized

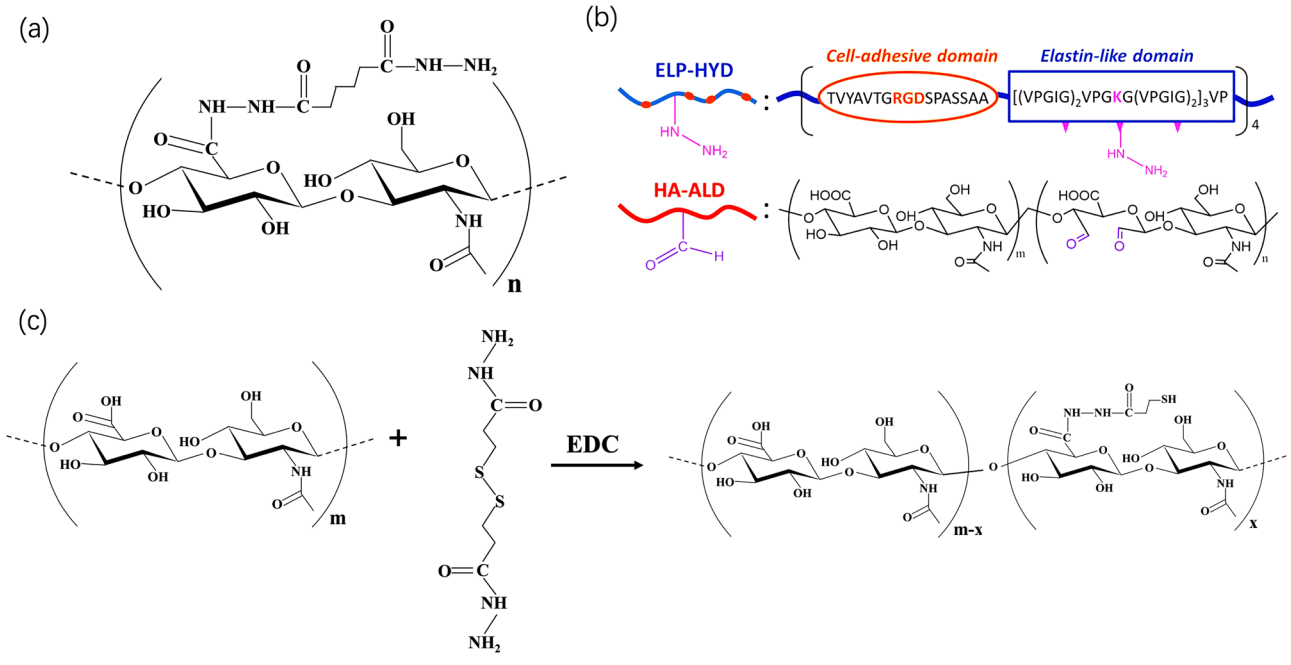

Figure 2: (a) The chemical structure of hydrazide-functionalized HA. (b) The structure of hydrazine-modified elastin-like protein and aldehyde-modified HA [56]. (c) Synthetic route of thiol-modified HA.

HA not only has the basic biological functions of HA but also can further bind with various polypeptides to enhance osteogenic differentiation [58]. Additionally, compared with HA, hydrazide-functionalized HA has better physicochemical properties and drug resistance.

3.1.2 Thiol modification

Thiol groups can be introduced to HA by using dihydrazide reagents containing a disulfide bond in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride [59] (Figure 2c). Hosack et al. utilized thiol-modified HA, gelatin, and heparin to fabricate a hydrogel system preloaded with platelet-derived growth factor (PDGF), keratinocyte growth factor, angiopoietin-1, and VEGF [60]. After being implanted into the Balb/c mouse ear pinna, the results *in vivo* found the new microvessel development and maturation at 7 days postsurgery [60]. Additionally, thiol-modified HA can crosslink spontaneously to form a hydrogel, which could be used to fabricate tissue-engineered bone scaffolds with porous structure and sponge-like properties after the process of freeze drying [61]. Moreover, Kazemirad et al. utilized thiol-modified HA and gelatin to construct new synthetic ECM hydrogels, whose mechanical properties could be altered by changing concentrations of the constituents [62]. Multifunctional electrophiles, such as poly(ethylene glycol) diacrylate, can be crosslinked with thiol-modified HA to improve the biocompatibility of hydrogel [63]. Additionally, thiol-modified HA can combine with ECM proteins and improve cell survival and differentiation [64].

3.1.3 Tyramine modification

Tyramine-modified HA was synthesized by amide bond formation between amine groups of tyramine and carboxyl groups of HA [65]. The synthetic route of tyramine-modified HA is shown in Figure 3a. Kim et al. synthesized tyramine-modified HA by *N*-hydroxysulfosuccinimide and 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide and utilized radical crosslinking reaction with H_2O_2 and horseradish peroxidase to fabricate hydrogel [66] (Figure 3b). Many osteogenesis-related cells and factors can be incorporated into tyramine-modified HA hydrogel for bone regeneration. Zhang et al. constructed an injectable hydrogel system loaded with BMSCs and bone morphogenetic protein-2 for bone repair and regeneration by enzymatic *in situ* crosslinking of HA-tyramine and chondroitin sulfate-tyramine in the presence of hydrogen peroxide and horseradish peroxidase [67] (Figure 3c). The results demonstrated that the injectable hydrogel system promoted bone regeneration [67]. The platelet lysate obtained from the peripheral blood can enhance cell attachment, viability, and chondrogenic differentiation. In another study, Jooybar et al. utilized the same method to incorporate platelet lysate into a cell-laden injectable HA-tyramine hydrogel [68]. The results *in vivo* found that the cells attached and spread out in HA-tyramine hydrogel. Additionally, tyramine-modified HA itself can also regulate MSCs' early attachment and behavior by activating related signal pathways [66]. However, there are still few studies focusing on the direct application of tyramine-modified HA to bone repair, and its specific bone repair mechanisms still need further research.

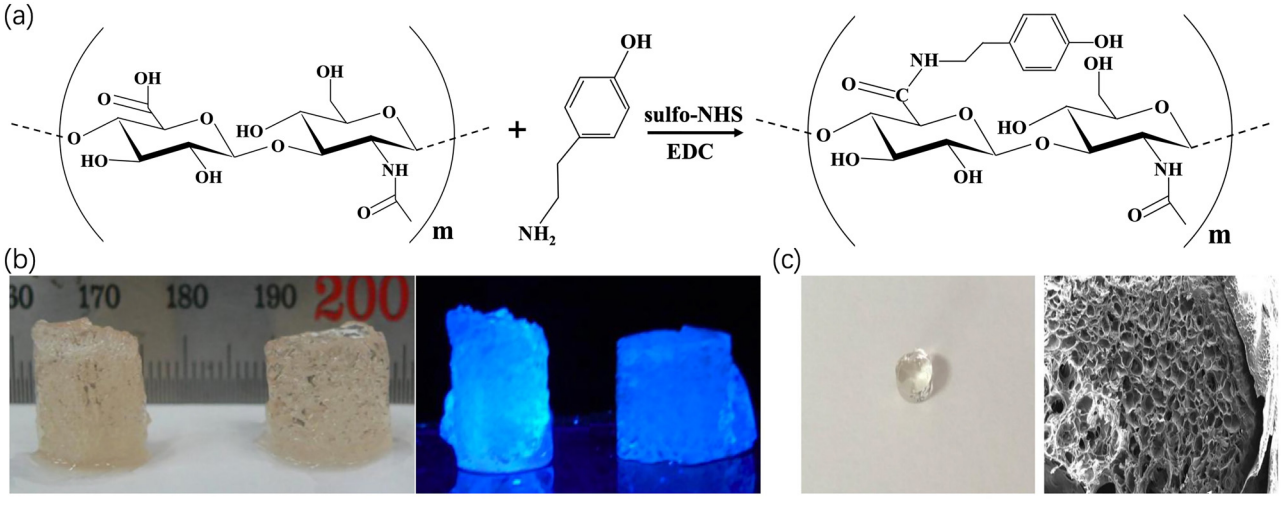

Figure 3: (a) The synthetic route of tyramine-modified HA. (b) The tyramine-modified HA hydrogels with and without exposure to UV light [66]. (c) The hydrogels composed of HA-tyramine and chondroitin sulfate-tyramine [67].

After chemical modification, the chemical and mechanical properties of HA can be enhanced, while its native biological functions can be maintained. Additionally, chemical modification of HA can also alter its resistance against enzymatic degradation and delay aqueous dissolution. Currently, hydrazide modification has been widely used in the chemical modification of HA because of its high cytocompatibility, efficiency, simplicity, reversibility, and mild reaction conditions [69]. In addition, hydrazide modification does not lead to cleavage of the HA chain. The thiol modification of HA has a high yield, efficiency, and a fast reaction rate. Compared with other kinds of chemical modification, thiol modification has a higher specificity [70]. Tyramine modification has advantages of mild reaction condition and a fast gelation rate. However, there are few studies focusing on the difference between various chemical modifications. Thus, the difference between various chemical modifications still needs further research.

3.2 Crosslinking of HA

Currently, crosslinking by means of radical polymerization has been widely applied in the *in situ* formation of HA-based hydrogels in the field of bone regeneration. Radical polymerization can react in the presence of aqueous solutions and control the chemical reactions [71]. Radical polymerization involves the placement of liquid biomaterials in the region of interest and its subsequent conversion into a solid implant, in the presence of crosslinking agents and some initiation source [72]. The initiators, such as photoinitiator in the presence of light, redox pair, and temperature, can induce the formation of radicals, which can react with reactive groups on the HA to form kinetic chains. Among these initiators, photoinitiated polymerizations are the most commonly used methods owing to their temporal and spatial control by light-triggered polymerization on the hydrogel formation. The biological properties of HA-based hydrogels can be adjusted by varying the molecular weight of HA, the concentration of the macromer, and the degree of methacrylation. In addition, photoinitiated polymerizations can also be applied in the direct cellular or protein encapsulation via altering radical concentrations or light intensities at a mild initiation condition. Currently, methacrylates degraded from methacrylic anhydride and glycidyl methacrylate in aqueous media are the most commonly used groups for HA-based hydrogel preparation.

3.2.1 Crosslinking with glycidyl methacrylate

Glycidyl methacrylate can react with HA to form a methacrylated HA (MeHA). Postoperative adhesions often remain permanent and complicate otherwise successful surgeries by tethering tissues together that are normally separated. An ideal adhesion barrier should prevent unwanted adhesions. Li et al. synthesized glycidyl methacrylate-hyaluronic acid (GMHA) conjugates by interacting glycidyl methacrylate with HA at room temperature, and then graft GMHA onto the surface of biomedical elastomer [73]. The result demonstrated that the GMHA could resist platelet adhesion and improve the proliferation of murine osteoblastic cell line MC-3T3-E1. In another study, Mayes et al. utilized alginate and GMHA to fabricate a robust hydrogel film, which showed a good ability of anti-adhesion in a rat peritoneal abrasion model for adhesion formation [74] (Figure 4a). Ma et al. utilized GMHA and poly(*γ*-glutamic acid) to fabricate an injectable hydrogel, which showed excellent anti-compression ability and outstanding shape recovery capability [75]. Moreover, researchers can modulate the mechanical properties of hydrogel by varying the ratio of poly(*γ*-glutamic acid) and GMHA. Many researchers incorporated cell or growth factors into GMHA for tissue engineering. Zhang et al. incorporated BMSCs and BMP-2 into GMHA to form hydrogels by photoinitiated polymerizations for bone regeneration [76] (Figure 4b). In another study, Khoshakhlagh et al. employed GMHA and puramatrix to form a structurally adjustable interpenetrating network system [77]. Hsieh et al. constructed a tissue-engineered scaffold for bone regeneration by using methoxy poly (ethylene glycol)-block-poly(ε-caprolactone), hydroxyapatite, and GMHA [78].

3.2.2 Crosslinking with methacrylic anhydride

The reaction of HA with methacrylic anhydride, first introduced in 1999 by Smeds et al., is another method to form an MeHA [80]. The networks of hydrogels formed from the MeHA macromer can be modulated by the concentration of the MeHA macromer, the number of reactive groups, and the molecular weight of HA [81]. Poldervaart et al. synthesized an MeHA hydrogel by methacrylic anhydride for 3D printable bone scaffold fabrication. The MeHA hydrogel exhibited good biocompatibility, excellent printability, and intrinsic *in vitro* osteogenicity [79] (Figure 4c). In another study, Ondeck et al. demonstrated that the mechanical property of MeHA hydrogels could be modulated by monomer concentration, duration of UV exposure, and methacrylate functionalization [82].

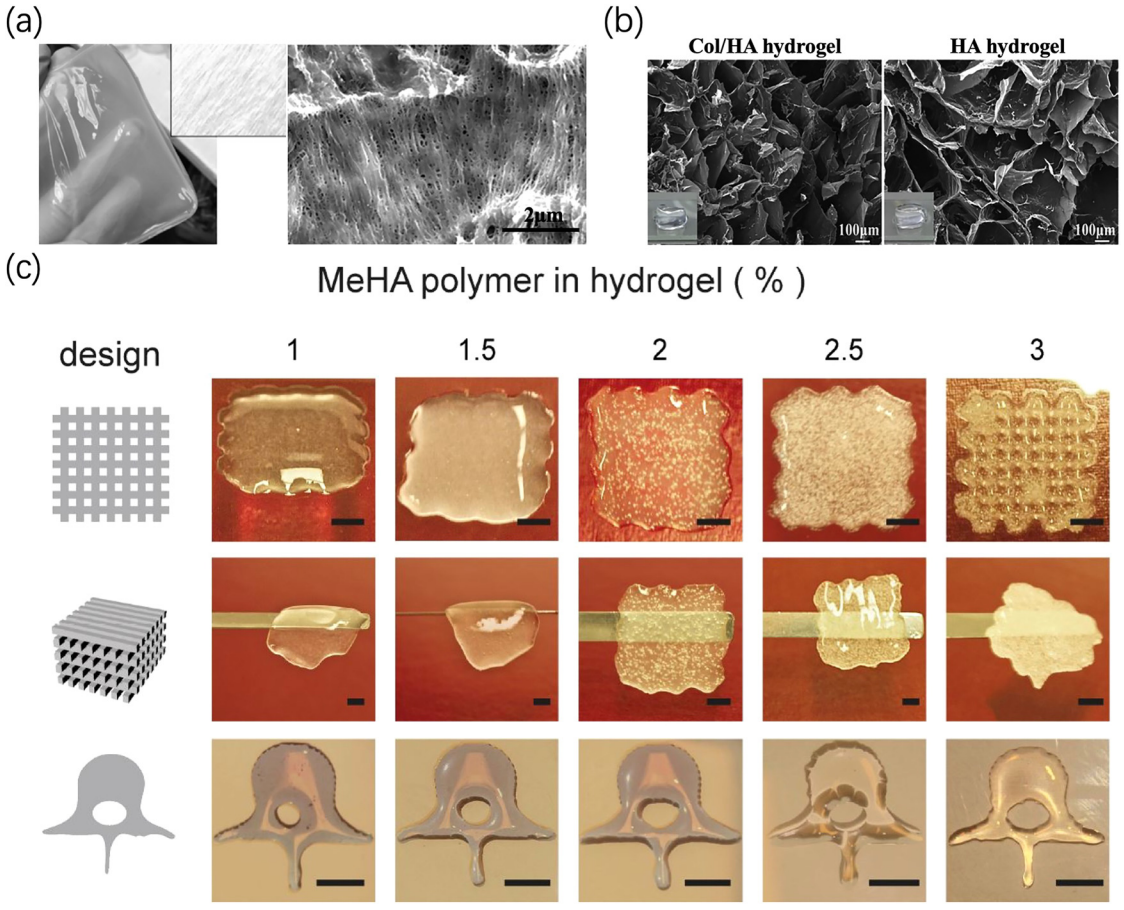

Figure 4: The GMHA showed a good printability and ability of anti-adhesion. (a) The hydrogel film composed of alginate and GMHA [74]. (b) GMHA hydrogel [76]. (c) The printability of MeHA [79].

Moreover, Zhang et al. found that MeHA hydrogels combined with adhesive peptide could enhance the proliferation and differentiation of encapsulated hiPSCs [83]. Additionally, the degree of methacrylation of HA by methacrylic anhydride can also regulate the encapsulated cell interactions. Therefore, it is crucial to evaluate the degree of methacrylation. Yousefi et al. demonstrated that infrared spectroscopy in attenuated total reflection mode could be an alternative method to evaluate the degree of methacrylation of HA hydrogels, including during the reaction progression [84].

4 Fabrication techniques of HA-based biomaterials

Currently, various processing techniques, such as phase separation, freeze drying, salt leaching, electrospinning, and 3D printing, have been applied for the fabrication of bone tissue-engineered constructs [21,22]. Bone tissue can be divided into compact bone tissue and spongy bone tissue. Additionally, the bone tissue in different parts has different shapes, mechanical properties, and microstructures. The biological characteristics of HA-based biomaterials were found to vary with porosity, interconnected network, and component composition. By combining with different processing techniques, HA-based biomaterials can be constructed with complex structures and various shapes for bone tissue regeneration. Therefore, this section will focus on various processing techniques used in the fabrication of HA-based biomaterials.

4.1 Freeze drying fabrication of porous scaffolds

Freeze drying, also known as lyophilization, is a dehydration process at a low temperature [85]. The process of

freeze drying includes freezing scaffolds, lowering pressure, and removing the ice by sublimation. The scaffolds can turn into porous constructs with interconnected micropore structure after freeze drying. Moreover, the porosities of scaffolds can be modulated via the rate of freezing. The constructs can keep their original shape and size during the process of freeze drying. Singh et al. synthesized a three-dimensional gelatin–hyaluronic acid–alginate (GHA) polymeric scaffolds by freeze drying for bone regeneration [86]. The results demonstrated that scaffolds had a high porosity, a rapid swelling behavior, and an interconnected pore morphology (Figure 5a). Additionally, porous scaffolds enhanced cell proliferation and induced osteogenic differentiation without external growth factors. Kaczmarek et al. developed 3D porous constructs based on chitosan, collagen, and HA by freeze drying, and then used them as matrixes for the calcium phosphate *in situ* precipitation for bone regeneration [87] (Figure 5b). Hu et al. developed biomimetic hybrid scaffold composed of HA, chondroitin sulfate, chitosan, and nano hydroxyapatite by freeze drying technology [88] (Figure 5c). The results showed that hybrid scaffold had hierarchical micro/nano structures and enhanced the proliferation and differentiation of osteoblasts. In another study, Li et al. fabricated chitosan–HA scaffolds containing nano-pearl powder by freeze drying for bone regeneration [89] (Figure 5d). The scaffold exhibits a high porosity. In another study, freeze drying technique was used to construct a tissue-engineered scaffold composed of chitosan, collagen, and HA supplemented with nano-hydroxyapatite for bone regeneration [90]. The *in vivo* results showed satisfactory tissue response on the implanted scaffolds.

4.2 Salt leaching fabrication of porous scaffolds

The salt leaching technique is a common method to fabricate porous scaffolds in the field of tissue engineering. The salt leaching technique involves mixing a polymer solution with solid salt particles of a definite diameter. The salt particles were leached after soaking in water, resulting in porous tissue-engineered scaffolds with high porosity [91]. Palumbo et al. utilized a hydrophobic/amino-functionalized derivative of HA to fabricate a porous scaffold by salt leaching technique [92]. The porosity of scaffolds can be modulated by altering the diameter of salt particles. However, salt leaching technology has the

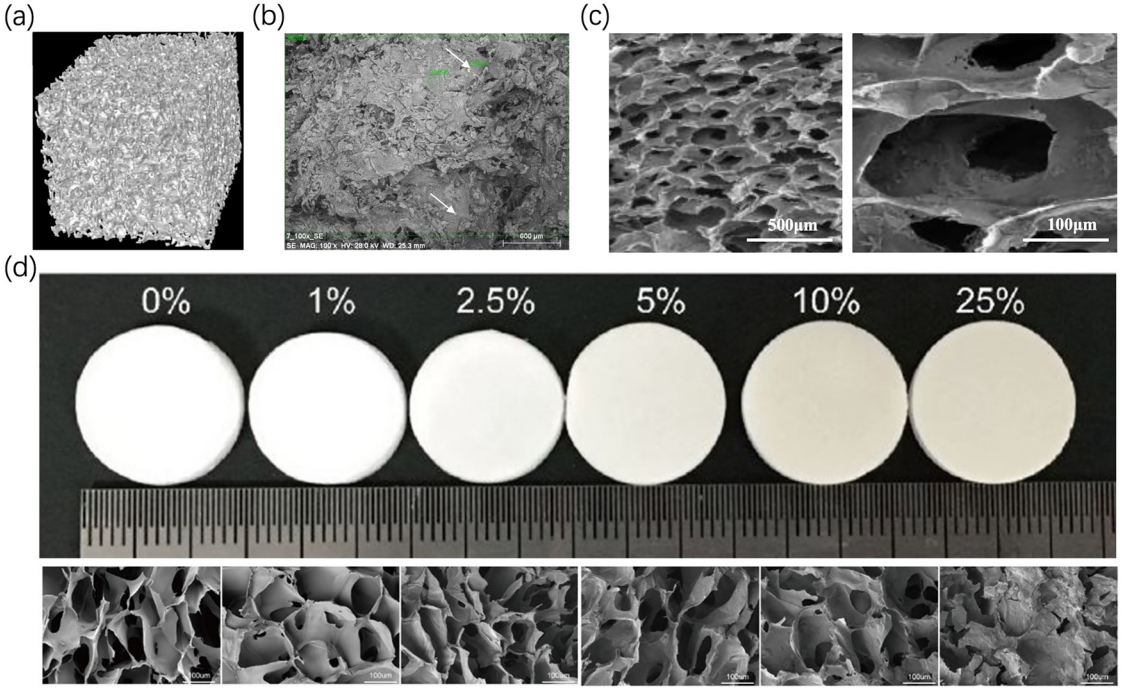

Figure 5: Various HA-based scaffolds can be constructed through freeze drying. (a) The 3D reconstruction image of GHA polymeric scaffolds [86]. (b) The SEM images of chitosan/collagen/HA scaffolds with precipitated calcium phosphate [87]. (c) The SEM images of HA/chondroitin sulfate/chitosan/nano hydroxyapatite hybrid scaffold [88]. (d) The photograph of nano-pearl powder/chitosan/HA scaffolds with different proportions of nano-pearl powder [89].

disadvantages of poor interconnection and abnormal pore shape.

4.3 Phase separation fabrication of porous scaffolds

The thermally induced phase separation (TIPS) technique is the creation of two distinct phases from a single homogeneous mixture [93]. TIPS technique is the most commonly used phase separation technology and involves the utilization of a solvent with a low melting point. Following cooling below the solvent melting point and subsequent removal of crystals by sublimation, a porous tissue-engineered scaffold is obtained [94]. TIPS technique has been widely used in recent years because of its potential to produce highly porous scaffolds with interconnected pore morphology. Erickson et al. fabricated bilayer chitosan–HA scaffolds using TIPS [95]. The results showed that the bilayer scaffold can promote the cell proliferation and migration of osteoblast-like cells. Additionally, the porosity and mechanical properties can be modulated by altering polymer solution concentration, quenching rate, and quenching temperature [96]. In another study, Jensen et al. fabricated a tissue-engineered scaffold consisting of polycaprolactone, HA, and β-TCP by TIPS [97]. The results showed that the scaffold could enhance cell migration and osteogenic differentiation *in vitro*.

4.4 Electrospinning fabrication of porous scaffolds

Electrospinning, a spinning technique, has been used for many years to generate nanofibers by utilizing electric force to draw charged threads of polymer solutions. Electrospinning originated from the textile industry [98]. Over the years, electrospinning has been gradually applied to the construction of tissue-engineered fibrous scaffolds which can mimic both the natural form and function of the ECM. Additionally, the characteristics of fibrous scaffolds can be modulated by varying the processing parameters of electrospinning [99]. Electrospinning can be used to fabricate nanometer-sized materials with the complex inner structure to mimic different types of ECMs. As one of the natural polymers, HA and HA derivatives are good candidates for electrospinning due to their biocompatibility and unique biological functions. However, HA alone cannot be used for electrospinning owing to its high viscosity at very low polymer concentration. Therefore, in most studies, HA is mixed with other polymers for electrospinning. Sujana et al. fabricated a biocompatible poly(L-lactic acid)-*co*-poly(ε-caprolactone)-silk fibroin–hydroxyapatite–HA nanofibrous scaffold by electrospinning [100] (Figure 6a). The results demonstrated that nanofibrous scaffolds could enhance osteoblast proliferation, osteogenic differentiation, and mineralization. Li et al. utilized HA oligosaccharides and collagen to fabricate a biomimetic mineralized nanofiber network which could enhance cell proliferation and induce osteogenesis *in vitro* [101] (Figure 6b).

4.5 3D printing fabrication of porous scaffolds

3D printing technology is the fabrication of a three-dimensional object, typically layer by layer, from a computer-aided design model or other geometrical data, such as X-ray imaging, magnetic resonance imaging, ultrasound imaging techniques, and computerized tomography scan [102–104]. 3D bioprinting involves the utilization of 3D printing-like techniques to combine cells and other bioactive molecules to fabricate biomimetic tissue constructs complex 3D architecture [43,105,106]. As one of the latest biotechnologies, 3D bioprinting has been widely used in bone tissue regeneration to fabricate bone tissue-engineered constructs with geometrically defined structures for personalized patient-specific therapy. 3D bioprinting technology has the advantages of precise spatiotemporal control on the distribution of cells, small molecules, osteogenesis-related growth factors, miRNA, and osteoinductive drugs [107]. Moreover, in the process of 3D bioprinting, bioinks play a crucial role in providing a stable 3D architecture for cells and mimicking the tissue niche *in situ* [108]. HA and HA derivatives can be used as bioinks for 3D bioprinting due to their biocompatibility and ability to regulate encapsulated cell behaviors.

Currently, several studies have used HA or HA derivatives as bioinks for the fabrication of tissue-engineered bone constructs. Noh et al. synthesized a hybrid hydrogel from HA, hydroxyethyl acrylate (HEA), and gelatin-methacryloyl and used it as a cell-laden bioink for bone regeneration [109]. The results demonstrated that the hybrid hydrogel showed excellent biocompatibility and good printability (Figure 7a). In another study, Wei et al. developed a composite bioink consisting of HA, silk fibroin, gelatin, and tricalcium phosphate and fabricated tissue-engineered

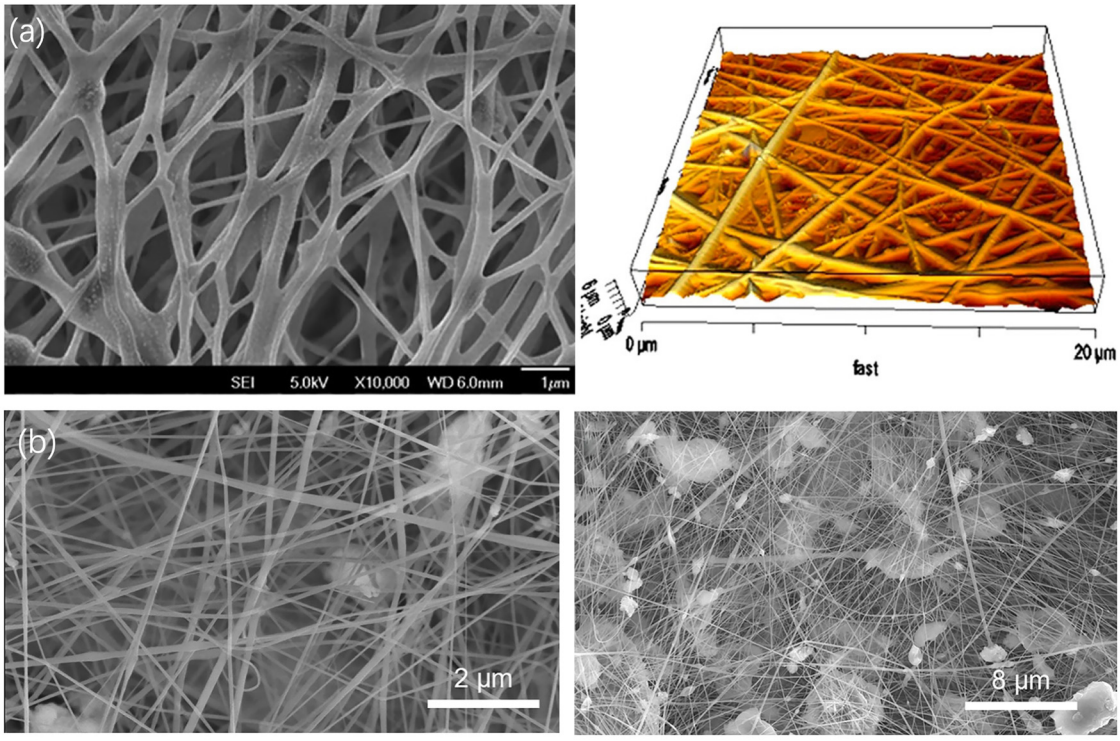

Figure 6: Various HA-based scaffolds constructed by electrospinning. (a) The SEM and 3D AFM of poly(L-lactic acid)-*co*-poly(ε-caprolactone)-silk fibroin–hydroxyapatite–HA nanofibrous scaffolds [100]. (b) The SEM images of nanofibers composed of HA oligosaccharides and collagen [101].

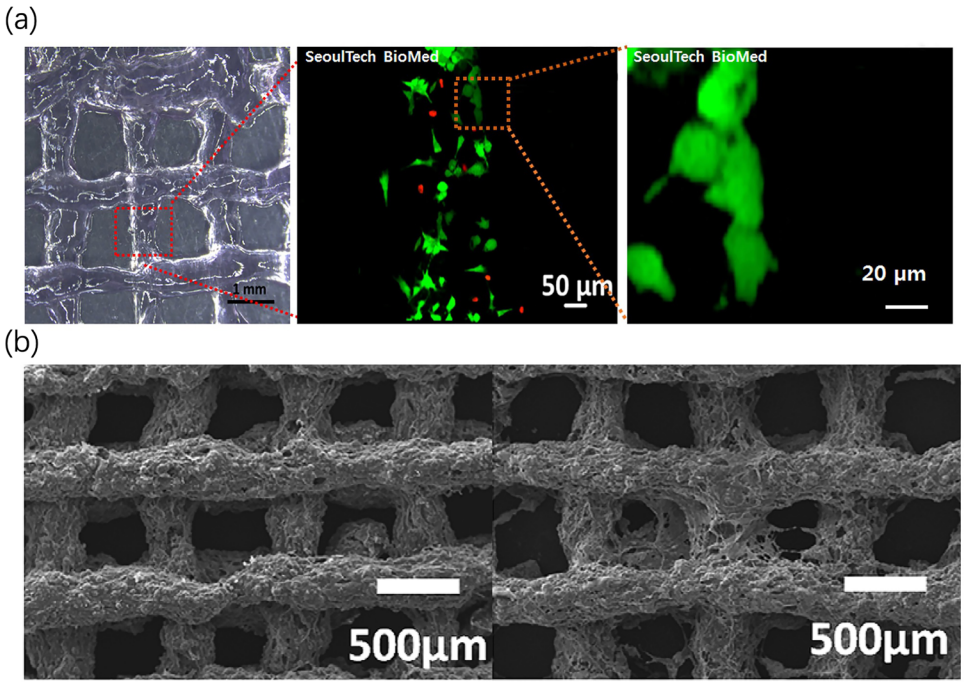

Figure 7: (a) Live and dead assay results of hybrid hydrogel by HA, HEA, and gelatin-methacryloyl [109]. (b) A bioink consisting of HA, silk fibroin, gelatin, and tricalcium phosphate treated with/without the platelet-rich plasma [110]. The HA-based bioinks showed excellent biocompatibility and good printability.

bone constructs coated by human platelet-rich plasma (Figure 7b) [110]. Müller et al. developed a hybrid bioink for bone tissue engineering, consisting of HA methacrylate, hydroxyapatite particles, and gelatin gum methacrylate. The results found that bioink displayed a strong interlayer adhesion between two printed ink layers. In another study, Wenz et al. utilized the same method to synthesize a hydrogel to encapsulate primary human adipose-derived stem cells for tissue-engineered bone fabrication using 3D microextrusion bioprinting technology [111]. The results showed that bioink enhanced bone matrix development and remodeling. However, the structure of tissue-engineered bone construct bioprinted with HA in current studies is relatively simple, and no studies have yet to report osteogenic growth factors being incorporated in HA-derived bioinks during the process of 3D bioprinting.

5 Applications of HA in bone tissue engineering

5.1 HA surface coatings for bone tissue engineering scaffolds

In recent years, coating design on various biomaterials has been the focus in the field of biomaterial fabrication. HA and its derivatives have been widely applied in coating fabrication of tissue-engineered bone scaffolds, such as metallic, polymeric, or ceramic surfaces. During the process of bone repair, various coatings on the tissue-engineered bone scaffolds are designed to induce favorable cell and tissue responses between the implants and surrounding host tissue [112]. As a natural ECM component, the specific biophysical properties of HA distinguish it from other components of ECM. Many technologies such as plasma spray, electrochemical technique, or polyelectrolyte multilayers are used to construct surface coating of tissue-engineered bone scaffolds [113].

5.1.1 Osteogenic functional coatings

Currently, many dental or orthopedic implants are made of alloys or ceramic owing to their excellent corrosion resistance and good hard-tissue compatibility [114]. However, poor osseointegration between implants and surrounding bone tissue can result in implants or prosthesis loosening [115]. The coating of dental or orthopedic implants can enhance the contact between host bone tissue and implants, reducing the risk of implant loosening and adverse reactions [116]. As one of the important components of the extracellular bone matrix, HA can promote the osseointegration of metallic and hydroxyapatite implants by modulating cell migration, adhesion, and differentiation. Song et al. developed HA/chitosan multilayer loaded with icariin onto the surface of the titanium implant via a layer-by-layer self-assembly system [117]. The results showed that the coating enhanced the proliferation, viability, and adhesion of osteoblasts and also promoted early osseointegration *in vivo* (Figure 8). Mathews et al. fabricated a tripolymer coating with chitosan, collagen type 1, and HA [118]. The results showed that the tripolymer coating enhanced mineralization and osteoblast differentiation. Moreover, HA-based coatings can be functionalized by the incorporation of growth factors and drugs. In another study, Aebli et al. fabricated an HA coating loaded with BMP-2 onto the surface of hydroxyapatite implants to promote bone formation around the implants [119].

5.1.2 Antibacterial functional surface coatings

Bacterial contamination at implant insertion can also result in dental implant failure [120]. However, treatment for infections around implants remains a challenge for dentists. The gold standard treatment for infection around implants is anti-infective therapy combined with implant replacement [121]. Therefore, antibacterial adhesion to the surface of the implants and reducing the risk of infection around the implants are of interest in the field of biomaterials. Currently, HA coating has also been used as a local antimicrobial or anti-adhesive barrier of implants. Valverde et al. fabricated multilayer antibacterial coatings as reservoirs of antibacterial agents onto modified titanium surfaces by using HA and chitosan [122]. The results showed that multilayer antibacterial coatings exhibited a good antibacterial property. Guarise et al. fabricated dopamine-functionalized sulfated HA coating for titanium implants [123]. The results showed that dopamine-functionalized sulfated HA coating could prevent biofilm formation and enhance osseointegration.

5.2 Osteogenic activity carriers

5.2.1 Carrying osteogenesis-related cells

Bone regeneration is a dynamic process that involves various cells, such as osteoblasts, MSCs, and ECs. MSCs

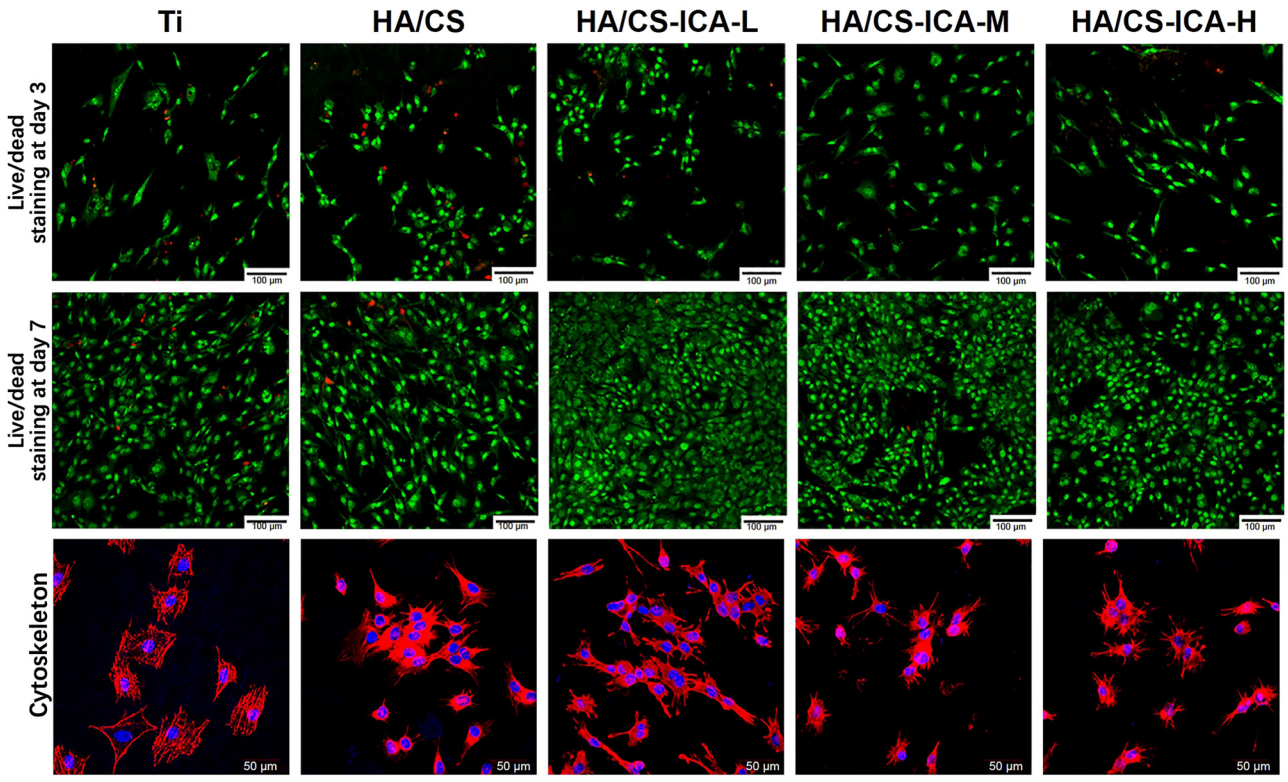

Figure 8: Live/dead and cytoskeleton staining of MC3T3-E1 cells on different surfaces of HA/chitosan multilayer coating [117].

are the most widely used type of osteoblasts in bone regeneration. MSCs come from a wide range of tissues, such as bone marrow, adipose, and umbilical cord [124]. Various HA-based hydrogels have been fabricated for cell delivery, owing to their biocompatibility and biodegradability [125,126]. Currently, many researchers utilize injectable HA-based hydrogels to encapsulate cells for bone regeneration by the minimally invasive method of injection. Rezaeeyazdi et al. developed an injectable hybrid hydrogel consisting of HA and gelatin [127]. The results showed that the hybrid hydrogel provided a suitable environment for cell attachment, spreading, and survival for osteoblasts (Figure 9a). In another study, Liao et al. fabricated a biocompatible thermo-gelling hydrogel scaffold, HA-*g*-chitosan-*g*-poly(*N*-isopropylacrylamide), to encapsulate stem cells for bone regeneration [128]. Moreover, HA-based hydrogels can also be used for multicell type delivery. Blood vessel formation involves the migration and proliferation of ECs and plays an important role in the process of bone regeneration [129]. Kang et al. constructed a hybrid hydrogel composed of collagen and HA as cell carriers for coculture of human adipose-derived stem cells and human umbilical vein ECs [130] (Figure 9b). In another study, Wenz et al. improved vasculogenesis and bone matrix formation through the coculture of ECs and stem cells in HA-based hydrogels [131].

5.2.2 Carrying osteogenesis-related growth factors

Growth factors are often a key component in the field of tissue engineering. Various growth factors play an important role in the process of bone regeneration and remolding [132]. Many growth factors alone or combined with scaffolds have been used for bone regeneration, including bone morphogenetic proteins (BMPs), fibroblast growth factors, VEGF, transforming growth factor, insulin-like growth factor, PDGF, and hepatocyte growth factor. Various growth factors can modulate the proliferation, differentiation, and recruitment of osteogenic-related cells to enhance bone formation [133]. However, the growth factor-based therapies for bone regeneration are limited by dosage-related adverse effects. Moreover, the delivery system of growth factors can also affect their efficiency, causing growth factors to be intrinsically less stable than other inorganic components, such as hydroxyapatite [134]. Therefore, how to incorporate the growth factors into the scaffolds while preserving the structural and functional integrity of the growth factors is still a challenge for researchers. As one of the natural polymers, HA is versatile for incorporating growth factors by cross-linking reaction. One common use of HA hydrogels is to accomplish spatiotemporal control of growth factor

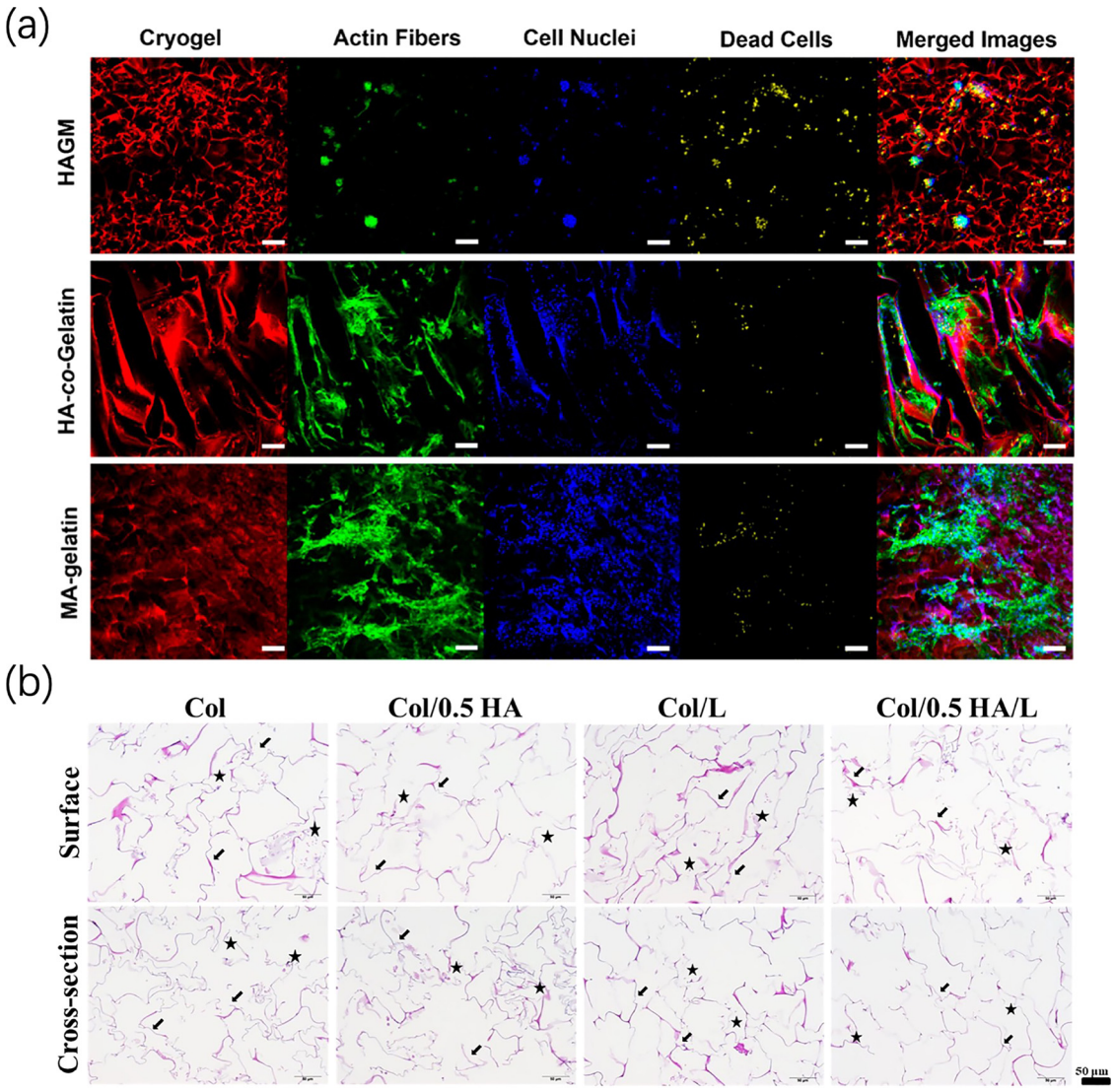

Figure 9: (a) Cell viability of hybrid hydrogel [127]; (b) hematoxylin and eosin stain of coculture of human adipose-derived stem cells and human umbilical vein ECs for 28 days in hybrid hydrogels (★: cells, ↙: scaffolds) [130].

release, owing to their biocompatibility, degradability, and non-immunogenicity. As a result, many researchers used HA and HA derivatives as a vehicle for growth factor delivery in bone regeneration. Xu et al. constructed heparin-decorated, HA-based, microscopic hydrogel particles (HGPs) for the controlled release of BMP-2 [135] (Figure 10a). The results confirmed that the addition of heparin to HGPs significantly improved the loading of BMP-2 and prolonged the release of BMP-2. In another study, Jha et al. fabricated BMP-2-loaded HA-based HGPs for growth factor's sustained release [136] (Figure 10b).

According to the types of growth factors delivered by HA and HA derivatives for bone regeneration, growth factors' delivery can be divided into single growth factor delivery and multiple growth factor delivery. Currently, most studies have used the method of single growth factor delivery to promote bone regeneration, while there are several other studies that used the combination of multiple growth factors with different functions to enhance bone formation [137–139]. Holloway et al. delivered stromal cell-derived factor-1α (SDF-1α) in combination with BMP-2 using proteolytically degradable HA hydrogels in a critical-sized calvarial defect [140]. The results *in vivo* demonstrated that SDF-1α and BMP-2 synergistically enhanced bone formation (Figure 10c). However, there is no consensus that multiple growth factors can enhance bone formation due to recent studies that have shown that the combination of BMP-2 and VEGF did not show a synergistic

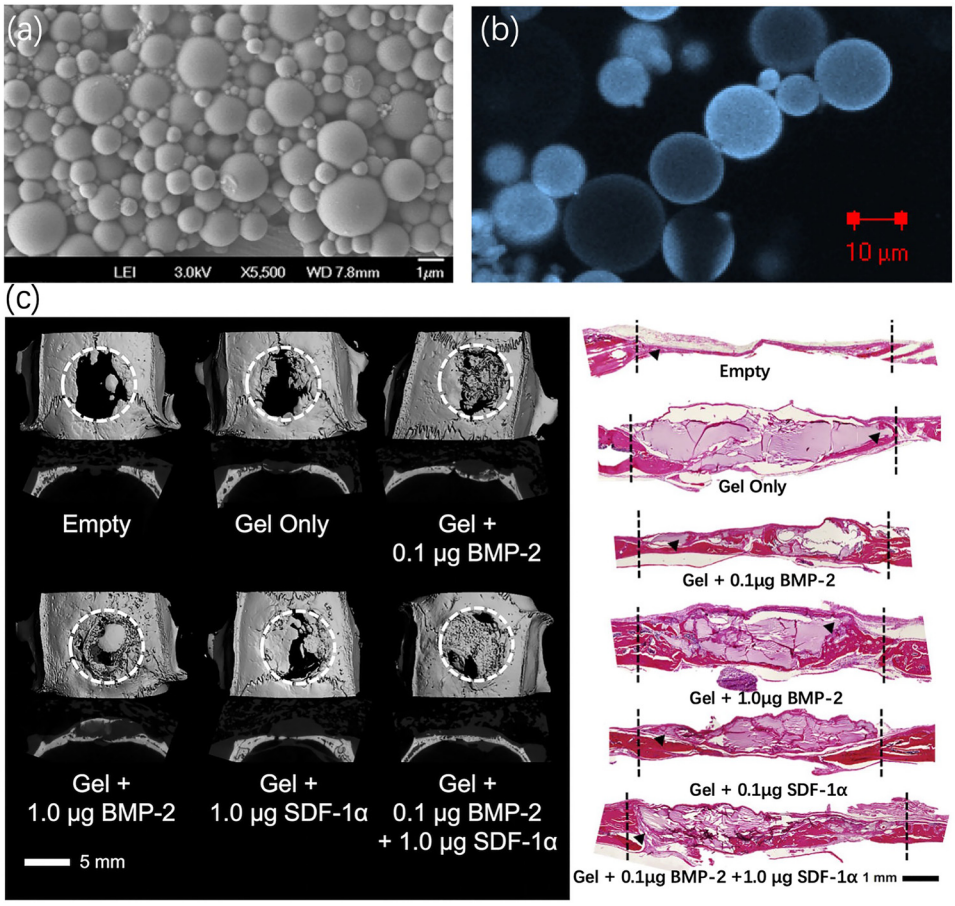

Figure 10: (a) The SEM of heparin-decorated, HA-based HGPs [135]. (b) The Cascade blue staining of BMP-2-loaded HGPs by HA [136]. (c) The μCT reconstructions and hematoxylin and eosin staining of calvarial defects for all treatment groups at 6 weeks [140].

or additive effect in a canine model of maxillary alveolar bone defects [141].

How to achieve a sustained and controlled release of growth factors is another important issue in growth factors' application for bone regeneration [133,142]. An ideal delivery system of growth factors should have advantages of adequate biodegradability, high biocompatibility, low toxicity, mechanical congruency, cost-effectiveness, ease of manufacture, and malleability [143]. In recent years, many HA-based biomaterials have been fabricated and used as delivery carriers to prolong the promotion effects of growth factors. According to the composition, all these HA-based biomaterials for growth factor delivery can be divided into single and hybrid HA-based biomaterials. For single HA-based biomaterials, the release of growth factors can be prolonged by altering the molecular weight and solution concentration of HA [144,145]. Additionally, chemical modification and crosslinking of HA in hybrid HA-based biomaterials are utilized to prolong the release period of growth factors [146,147]. HA can be combined with other components, such as chitosan, alginate, and collagen, to form hybrid HA-based biomaterials for growth factor delivery [148]. Nath et al. fabricated a hybrid hydrogel with chitosan and HA as a carrier for the controlled release of BMP-2 [149]. The results showed that hybrid hydrogel achieved a sustained release of BMP-2 for more than 1 month *in vitro*. In another study, Chung et al. fabricated a self-assembling collagen–HA membrane to deliver BMP-2 for bone regeneration [150]. The results found that the hybrid membrane achieved the sustained release of 17% of total loaded BMP-2 over the course of 49 days.

5.2.3 Carrying osteogenesis-related drugs and other components

A variety of osteogenesis-inductive drugs have also been delivered by using HA-based biomaterials. Lee et al. developed hollow microparticles composed of catechol-modified HA shell and silica core for drug carriers [151].

Bae et al. fabricated photo-cured HA hydrogels loaded with simvastatin for bone regeneration [152]. In another study, Schmidt et al. demonstrated that sulfated HA and dexamethasone possess a synergistic potential in the osteogenic differentiation of MSCs [153]. Moreover, various anti-osteoporosis drugs, such as risedronate, bisphosphonate, and zoledronate, have also been incorporated into HA-based biomaterials to enhance bone regeneration and peri-implant bone augmentation [154–156]. In addition to drug delivery, several studies have shown the potential of HA-based biomaterials for the delivery of inorganic osteogenesis-inductive components, such as hydroxyapatite, beta-tricalcium phosphate, and bioactive glass [157,158].

6 HA in bone regeneration nanomedicine

Nanomedicine is the medical application of nanotechnology. Currently, various tissue-engineered biomaterials with nanoscales such as nanoparticles and nanofibers have been fabricated by nanotechnology for bone regeneration [159]. Due to their biodegradability, biocompatibility, non-immunogenicity, and non-thrombogenicity, HA and its derivatives have been utilized to fabricate HA-based biomaterials with nanoscales for bone regeneration. Séon-Lutz et al. utilized the electrospinning technique to fabricate biomimetic nanofibrous scaffolds consisting of poly(vinyl alcohol) and HA [160]. In another study, Fischer et al. utilized collagen and HA to develop a nanofiber mesh by electrospinning for bone regeneration [161]. However, HA-based nanofibrous scaffolds constructed by electrospinning technology usually lack a large volume, which greatly limits its application for bone regeneration *in vivo*. Additionally, various nucleic acids have also been incorporated into HA-based nanoparticles for bone regeneration [162]. In addition, many miRNAs play important roles in the proliferation and differentiation of MSCs. Wang et al. constructed hybrid nanoparticles by chitosan and HA to deliver microRNA-21, which dramatically enhanced the osteogenesis in cell sheets of BMSCs [163]. In another study, Wu et al. utilized chitosan, tripolyphosphate, and HA to fabricate nanoparticles to deliver antimiR-138, which showed a positive effect of the MSC osteogenesis [164]. Curcumin (CUR) is a bright yellow chemical produced by Curcuma longa plants and can be used in the treatment for osteoporosis. Alendronate (ALN) can also be used to treat osteoporosis caused by menopause, steroid use, or gonadal failure. Dong et al. fabricated ALN/CUR nanoparticles decorated with HA by nanotechnology [165]. The results showed that HA-ALN/CUR nanoparticles could significantly enhance the proliferation, differentiation, and mineralization of osteoblasts *in vitro*.

7 Conclusion and future perspectives

HA and its derivatives have been utilized in the fabrication of tissue-engineered bone substitute for many years, due to their outstanding biodegradability and biocompatibility properties. The purpose of this study is to report the properties, processing, and applications of various HA-based biomaterials for bone regeneration in recent studies. As a matrix component of ECM, HA can activate several signaling pathways to modulate cell behaviors. HA and its derivatives can be chemically modified in various ways to improve their mechanical properties, bioactivity, and resistance against enzymatic degradation. By combining different processing and fabrication technologies, more highly structured HA-based biomaterials with diverse physiochemical properties can be constructed and used as a hybrid scaffold to repair different parts of bone tissue and promote bone regeneration. The hybrid scaffold exhibits excellent physicochemical and biological properties during the process of bone repair. In the field of orthopedics and dental implants, HA and its derivatives can be fabricated into coatings to promote osseointegration between the prosthesis and the surrounding bone tissue and prevent bacteria adhesion and biofilm formation. Moreover, by carrying different osteoinductive components, such as cells or growth factors, the osteogenic ability of HA-based biomaterials can be enhanced.

Although various HA-based biomaterials for bone regeneration have many advantages, there are still many challenges for researchers to face. Currently, there are few studies focusing on the molecular mechanisms of HA and its derivatives during the process of bone repair. Although the effects of HA and its derivatives on bone regeneration have been demonstrated by many studies, the differences in osteogenic ability between different HA derivatives still need further research. There are a lot of scientific research studies on HA and its derivatives as carriers of various osteogenesis-related cells and growth factors at present, but most of them are in the stage of laboratory research. And it is expected that the next few

years will see the practical realization of some of these ideas in clinical trials. Additionally, how to achieve sustained, controlled release of osteogenic miRNAs in HA-based carriers and the interaction between HA and encapsulated cells should also be further studied. Furthermore, the emergence and development of 3D printing technology has opened a new field of personalized HA-based biomaterial construction. With the continuous developments of tissue engineering and material fabrication technologies, more intelligent and complex HA-based biomaterials will likely become promising candidates for bone regeneration.

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