

Collagen-based biomaterials for bone tissue engineering

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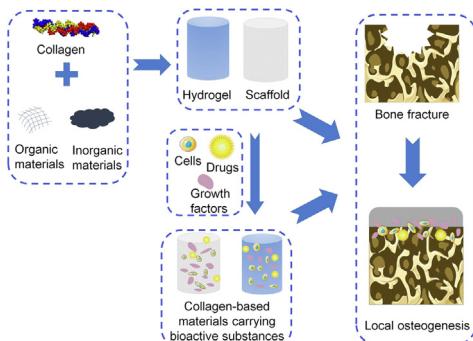
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HIGHLIGHTS

- Collagen based biomimetic materials are developing rapidly in bone tissue engineering.
- Extraction and processing of collagen.
- Mechanism of collagen promoting osteogenic differentiation.
- Synthesis of collagen-based hydrogels and collagen scaffolds.
- Collagen-based biomimetic materials carrying bioactive substances for repairing bone defects.

GRAPHICAL ABSTRACT



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ABSTRACT

In recent years, there has been increasing interest in using biomimetic materials for bone tissue engineering. However, the high immunogenicity and low mechanical properties of biomimetic materials limit their applications *in vivo*. As an essential component of the extracellular matrix in bone tissue, collagen is ideal because it easily degrades and has strong plasticity and low immunogenicity. However, pure collagen alone does not yield satisfactory experimental results. Therefore, biomimetic bone tissue engineering scaffolds are often prepared together with other bioactive materials to better replicate the bone tissue's biological microenvironment. To better understand the engineered scaffolds, we have summarized and analyzed current research in collagen-based biomimetic bone tissue engineering. This review focuses on the acquisition and processing of collagen, the mechanisms involved in collagen-induced osteogenesis, the biomimetic materials composed of collagen and different materials, and the application of collagen-based bioactive materials in bone tissue engineering.

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1. Introduction

The goal of bone tissue engineering is to develop novel bone substitutes to replace bone tissue defects and restore bone integrity [1]. As a natural solid biological composite material, bone has a unique multi-scale hierarchical structure, high strength,

and fracture toughness. In natural tissues, the extracellular matrix (ECM) comprises extracellular molecules secreted by cells, which provide spatial and mechanical cues to cells and physical support for tissues [2]. It not only serves as a benign scaffold for the arrangement of cells in connective tissue but also has a dynamic and flexible role in defining cell behavior and tissue function [3]. Therefore, it is a reasonable strategy to prepare a graft that simulates the damaged tissue or organ ECM and sequentially repairs it.

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As the main component of soft and hard tissues, collagen is the most common protein in mammals, accounting for about 1/3 of the body's protein tissue mass [4,5]. Collagen is widely distributed in various tissues, such as bones, skin, tendons, and teeth [6,7]. So far, 28 types of collagen have been identified, and type I collagen content is the most abundant in the ECM, especially in tendons and bones [8]. The various collagens have similar structures; specifically, there are three interwoven chains (α -polypeptide chains) involving homotrimers or heterotrimers. These structures are determined by collagen type, where each chain has a conformation similar to that of polyalanine II. The impregnable triple helix composition results from the repetitive Gly-X-Y triplet, where X is usually a proline (or any other amino acid), and Y is often hydroxyproline [9]. Glycine is required in every third position because of its small volume, capturing the triple helix center's limited space.

Previous studies have confirmed that collagen is a feasible biomaterial because of its excellent biocompatibility, degradability, adhesion, osteogenic induction properties, and low immunogenicity [10]. However, its poor mechanical strength in terms of physicochemical and mechanical properties limits its further applications. Using collagen alone for bone tissue engineering may not be appropriate. Therefore, it is necessary to modify collagen or combine collagen with a scaffold to improve its performance. Collagen is currently used for hydrogels or collagen-based scaffolds. Collagen provides an excellent matrix for many cell types and opens up the possibility for 3D cell culture using a hydrogel. However, previous studies have shown that collagen-based matrices are biologically active and stimulate cell migration in scaffolds [11]. Improved collagen can be incorporated into a titanium scaffold to provide mechanical support without affecting other properties [12].

In this paper, the application of collagen in bone tissue engineering is described in detail, with a specific focus on the extraction and separation of collagen, the mechanisms regulating bone regeneration, and related work (Scheme 1). Additionally, the challenges of collagen application in regenerative medicine are summarized to guide the clinical application of collagen.

2. Collagen structure, preparation and processing methods

Collagen consists of three polypeptide chains, known as collagen α chains, numbered in Arabic numerals. The three chains can be the same to form homotrimers or different to form heterotrimers. The three chains of collagen forming fibrils are three left-handed polyprotein II helices, which are twisted into right-handed triple helices, with a residue interlaced between adjacent chains. There is one glycine, high content of proline and hydroxyproline, interchain hydrogen bond and electrostatic interaction in every three residues, involving lysine and aspartic acid [13,14]. Three chains are associated with a residue shift to accommodate glycine residues in the kernel. Each type of collagen has a unique trimerization domain, which can select and arrange three specific chains [15]. For most collagen types, this domain is located in the non-triple helix domain at the carbon terminal. Four types of collagen trimerization domains have been reported: the NC1 domain of type IV collagen, the C1q-type domain of types VIII and X, the multiplexin trimerization domain of types XV and XVIII and the C-propeptide of fibrillar collagens (types I, II, III, V and XI) [16].

The extraction sources of collagen are very rich, and the main source is cattle [17]. Collagen can be obtained from different tissues of cattle, such as tendon, bone and lung tissue. In addition, pigs are also a common source of collagen. Porcine collagen is very similar to human collagen, so it has almost no immune restriction, and is often used for hernia repair and wound healing [18,19].

Table 1 summarizes the recent sources and methods for collagen extraction. Collagen is a molecule with low immunogenicity. When ingested or injected into a foreign body, it reduces the possibility of rejection. The only fragment that can induce immune response is located in the helical region and terminal peptide region of the chain [20]. Despite its low antigenicity, this molecule can be modified to eliminate any immune response. The immunogenicity of collagen can be reduced by protease specific removal of non-helical region of collagen molecules or introduction of cross-linking [21]. The function of cross-linking is to screen antigen sites and reduce antigen antibody reaction [22].

Natural collagen is a highly hydrophilic protein that is insoluble in organic solvents. Collagen is crosslinked by covalent bonds, which allow for the maintenance of the quaternary structure and prevent dissociation of molecules from its fibrous conformation. The amount of dissociated collagen is closely related to the extraction conditions used. Except for type I collagen, no other collagen type can be isolated from adult tissues under non-denaturing conditions [23]. Collagen molecules can be extracted and purified from tissues using a variety of techniques, such as acid treatment (usually dilute acetic acid), alkali treatment (usually sodium hydroxide solution), or protein hydrolysis procedures followed by neutral salt treatment, dialysis, precipitation, and centrifugation [24]. Metal nanoparticles can initiate physical and photochemical crosslinking when exposed to ultraviolet light [25,26]. The addition of polyvinylpyrrolidone-capped zinc oxide nanoparticles in the collagen matrix has previously been shown to induce gelation without chemical crosslinking agents [27].

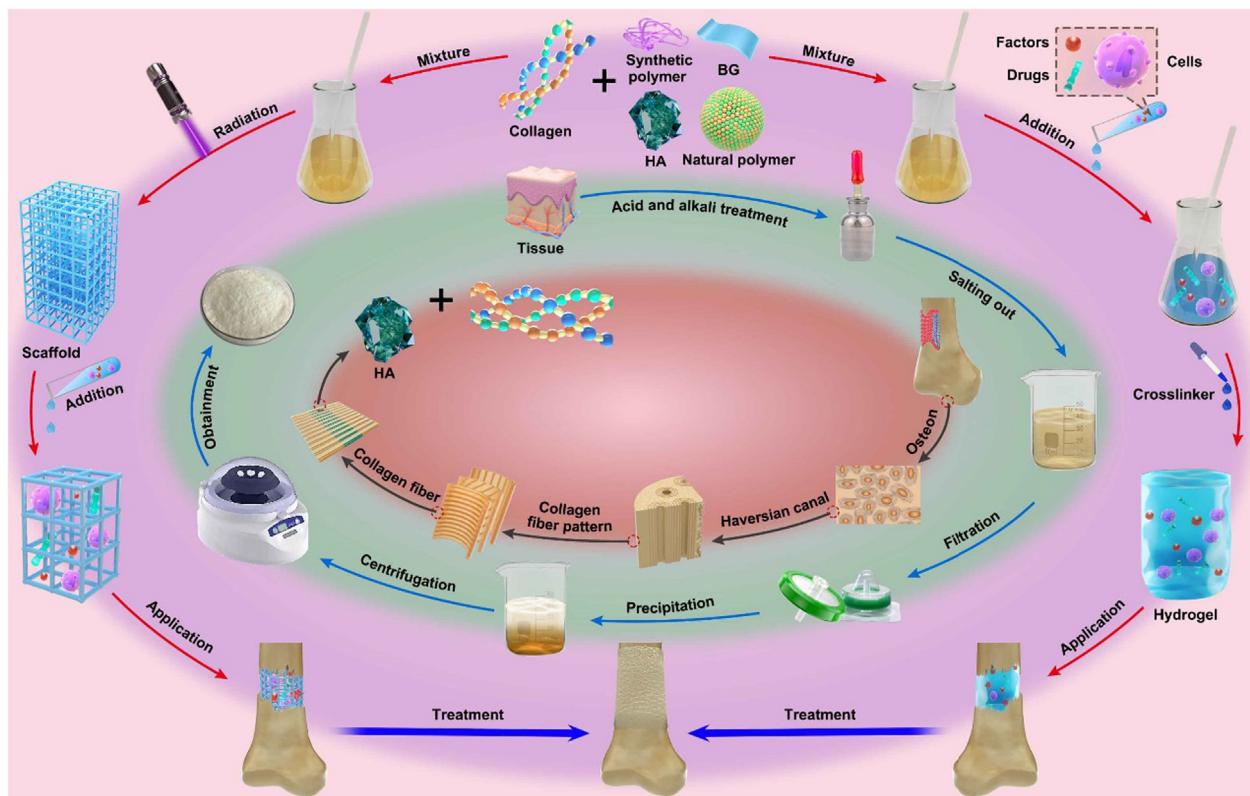
To achieve the specific functions required for tissue regeneration in scaffold applications, collagen (including crosslinked or mixed) must be processed according to specific tissue requirements. Various chemical agents or physical treatments can cross-link collagen to enhance mechanical and chemical stability. Aldehydes, such as glutaraldehyde and poly epoxy, are the most commonly used chemical reagents [28]. Physical treatments such as dehydration (heat treatment) and radiation (ultraviolet, gamma, and microwave) can effectively induce crosslinking. Chemical crosslinking performed using aldehydes, isocyanates, and carbodiimide can be used; however, it has been shown that this reduces biocompatibility [29,30]. Drugs can be incorporated into the collagen system by simple embedding, hydrogen, or covalent bonds. Additionally, changing the collagen structure through crosslinking can reduce its water absorption capacity and slow down the diffusion of any binding molecules [31]. Furthermore, the interaction between drugs and collagen can also prolong drug release.

3. Biological properties of collagen in bone tissue engineering

As an essential component of the bone matrix, collagen plays a crucial role in bone formation. The role of collagen in bone formation is not completely clear. Existing research shows that collagen can promote the proliferation and differentiation of bone mesenchymal stem cells (BMSCs) and osteoclasts, and the overall effect is the induction of osteogenesis. Mineralized collagen also has a unique role in accelerating bone regeneration.

3.1. Collagen participates in mineralization formation

Collagen molecules self-assemble in compartments of extracellular tissue spaces in intimate association into a two-dimensional periodic and overlapping structure that appears periodically. When the collagen molecules are arranged along the long axis, there will be a connecting structure composed of hydroxyapatite (HA) crystals between adjacent collagen molecules, which plays a role of fixation. HA crystals in the same plane are arranged in a straight line.



Scheme 1. (Center circle) Mineralization of collagen. (Middle circle) Extraction process of collagen. (Outer circle) Collagen in hydrogels and scaffolds for bone tissue engineering.

Table 1

The recent sources and methods of collagen extraction.

Species	Position	Type	Temperature	Methods	Productivity	Reference
Rat	Tails	Type I	-80 °C -20 °C	Lyophilization		[32]
Catshark	Skin		25 °C 4 °C	Response surface methodology	61.24%	[33]
Sheep	Bone, cartilage, Carcass trim-mings, meat	Type I	4 °C ±8°C	Constant agitation	12.5%	[34]
Pufferfish	Skin	Type I	28.4 °C	Electrodialysis	67.3%	[35]
Tilapia	Skin		4 °C 20 °C	Stir	19.0%	[36]
Jellyfish	Bell, Oral arms		4 °C	Acid assisted extraction, Pepsin assisted extraction, Physical disruptions	70%	[37]
Loach	Skin	Type I	4 °C	Salted out	22.42% (ASC), 27.32% (PSC)	[38]
Yak	Rumen smooth muscle	Type I, Type III, Type IV, Type V, Type VI, Type VIII, Type XII		Enzymatic method	3.62%	[39]
Silver carp	Bone, Skin	Type I	4 °C -20 °C	Sequential hydrolysis	15.4%	[40]
Carp rohu	Bladder	Type I	4 °C	Enzymatic method	46.52%	[41]

The planar structure of collagen molecules is arranged in parallel on a plane. Multiple collagen planes are parallel and tightly bound together to form a certain curvature degree, but collagen molecules of different planes are not parallel but overlap at a certain angle [42,43]. Multiple collagen planes arranged in parallel are combined into bone and Haversian tubes, thereby completing the assembly of collagen molecules in the microstructure (Scheme 1). In addition to being used as a structural substitute for bone tissue, mineralized collagen can also rely on its bone conductivity and biological activation to promote bone regeneration.

In bone tissue, collagen fibrils become hard through the integration of the mineral phase [44]. The existence of the organic phase in the structure increases the ultimate tensile strength by approximately two times and Young's modulus ten times. Although the mineral content may be higher, the mechanical properties of braided bone, which is composed of unstructured collagen fibers, are lower than that of the layered bone [45]. Studies have shown that bone strength is mainly determined by tissue mass and stiffness, where stiffness is determined by mineral phase, and collagen matrix mainly contributes to bone toughness [46–49]. The

decrease of mineral deposition can result in decreased bone stiffness and poor bone formation.

3.2. Collagen promotes osteogenic differentiation of BMSCs

Collagen activates osteogenic differentiation of BMSCs by activating two signaling pathways, extracellular-regulated protein kinases (ERK) and protein kinase B (PKB). Simultaneously, collagen can also indirectly activate ERK by activating Phosphatidylinositol kinase (PI3K) to enhance osteogenesis [50]. Additionally, integrins can promote the adhesion of early BMSCs. Tsai et al. tested whether ERK and PKB required integrin activation with blocking antibodies that interrupted integrins in cells [51]. As a result, alkaline phosphatase (ALP) staining was not significantly different from the control group, suggesting that collagen activated the ERK and protein kinase B (AKT) pathways without activating integrins. It was found that adhesion was directly proportional to the amount of integrin binding [52,53]. By interacting with $\alpha 2\beta 1$ integrin, collagen activates the mitogen-activated protein kinase (MAPK) pathway, then the relevant osteogenic genes, and finally, the cells differentiate into osteocytes [54–56].

3.3. Collagen inhibits osteoclasts differentiation

At present, it has been confirmed that four major integrins ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, and $\alpha 11\beta 1$) can regulate the adhesion of osteoclasts to the bone after binding with collagen [57,58] as well as form an acidic environment conducive to bone degradation [59]. Collagen has recently been shown to be a ligand in the leukocyte receptor complex family, including osteoclast-associated receptors (OSCAR) and leukocyte-associated Ig-like receptor-1 (LAIR-1) [60]. OSCAR is a stimulatory receptor activated by collagen and is particularly important for osteoclast differentiation. The binding of collagen to OSCAR results in the recruitment of immunoreceptor tyrosine-based activation motif (ITAM)-containing Fc receptor (FcR)- γ chains and the activation of calcium signals for downstream effects [61,62].

LAIR-1 is an inhibitory signal for osteoclast formation, which is activated by collagen. Its two ITAMs are phosphorylated to recruit protein tyrosine phosphatase 1 and protein tyrosine phosphatase 2. These phosphatases directly dephosphorylate Syk, zap70, and pIg γ , preventing ITAM-mediated stimulation of protein kinases and calcium signals [63,64]. LAIR1-mediated inhibition of osteoclastogenesis may help to prevent premature absorption of freshly placed osteoblast-like sites. The mechanism of collagen action on cells is shown in [scheme 2](#).

4. Collagen-based materials in bone tissue engineering

Hydrogel is a cross-linked highly hydrated colloid with three-dimensional network structure. It is a hydrated physical form of collagen-based materials and many other natural polymers. Because of its high moisture content, hydrogel have high permeability to oxygen, nutrients and metabolites, and have been applied in many fields, including tissue engineering scaffolds and local drug delivery. Hydrogel have also been used as adhesives, sealants and filling materials [65,66]. However, hydrogel cannot bear the pressure of bones, usually used to wrap cells or drugs, and collocation with other hard structures. The scaffold can provide the appropriate hardness to bear the pressure of the fracture site. As one of the most commonly used scaffolds materials, collagen usually lacks strong mechanical properties, therefore the addition of other components is still one of the most commonly used strategies to improve the mechanical strength and osteogenic ability of collagen scaffolds.

For decades, researchers have been trying to find a way to treat bone defects, instead of autologous bone transplantation, and hope to achieve better therapeutic effect. The following will specifically discuss the biomimetic graft synthesized by collagen and different materials.

4.1. Collagen-based hydrogel in bone regeneration

4.1.1. Raw collagen-based hydrogel

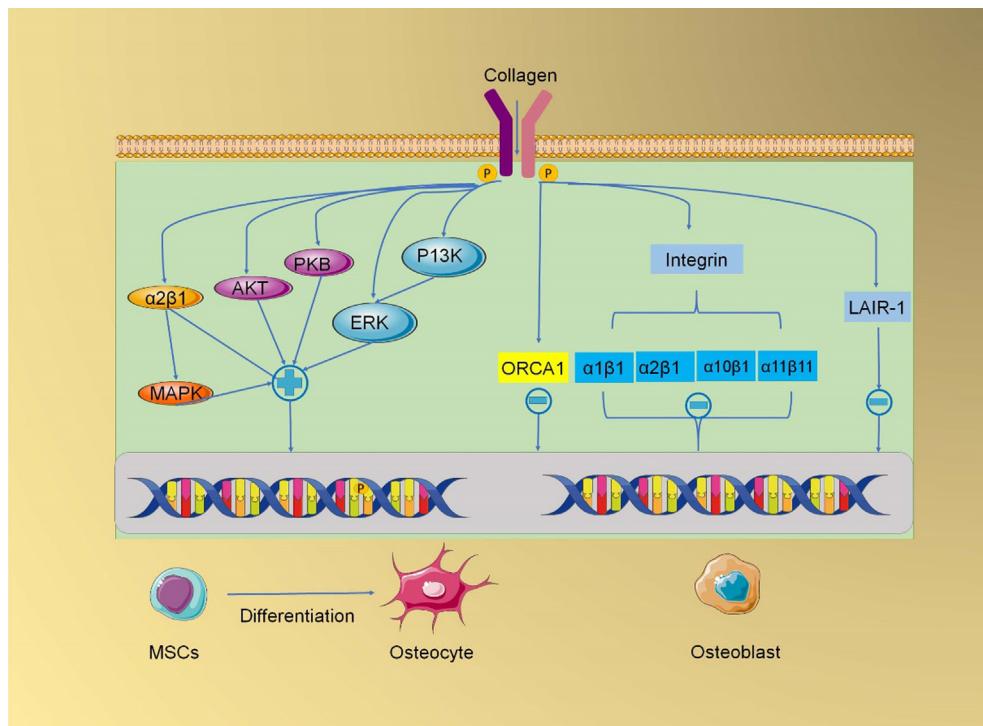
Collagen has been repeatedly demonstrated to maintain chondrocyte phenotype in 3D culture. Chondrocytes can also be evenly distributed in 3D space and synthesize ECM to form new cartilage-like tissue [67]. Ochi et al. used cartilage cells cultured in collagen hydrogel to treat knee cartilage defects [68]. The results showed that the boundary between the transplanted chondrocyte gel complex and the subchondral bone defect was not obvious, which means that the transplanted tissue had been steadily integrated into the adjacent subchondral bone. Katsube et al. found that within six months after surgery, the sites reconstructed with collagen gel-cultured chondrocytes had better histological scores than those reconstructed by suspension culture [69]. Each tissue has a unique ECM composition, and this can be customized according to its own specific physiological and mechanical requirements to maintain a specific cell phenotype and functionality [70].

Compared with dermal ECM, tendon ECM can provide a more similar environment to the natural tendon-bone interface (TBI) area and play a more appropriate role in the damaged TBI area. Kizawa et al. extracted collagen from human tendons and processed them into hydrogels to repair the tendon-bone interface nonunion after rotator cuff tear [71]. The results showed that the collagen content and bone mass used for repair were improved compared with the control group. When a proper connection is made between the tendon and the bone, the bone mass of the tendon insertion begins to recover.

A study of the cytoskeletal components of bone cells found that bone cells cultured in 3D exhibited more actin filaments compared with 2D culture [72]. In addition, the cells cultured in 3D showed amorphous mineralized granules, while the cells cultured in 2D showed mineralized nodules, which only showed the difference in volume ([Fig. 1](#)). Another study demonstrated that collagen's contraction during bone regeneration was related to osteoblast activity. In this study, gene expression was altered as a result of inherent mechanical stress [73]. In this process, osteocytes interact with the collagen fracture callus and then mineralize the matrix to promote fracture healing. BMSCs highly express type I collagen before the pre-coacervation (the initial stage of cartilage formation), and the expression is decreased during chondrocyte differentiation [74,75]. Studies have found that BMSCs cultured in collagen with the appropriate density can differentiate into cartilage. However, cells with low density did not become cartilaginous in the 3D collagen culture [76]. A series of gradient cartilage tissues produced by this culture system is very suitable for tissue engineering.

To further explore the effect of collagen shear stress on the osteogenic and angiogenic potential of BMSCs and human umbilical vein endothelial cells (HUVECs), Bao-Ngoc et al. used a collagen hydrogel to co-culture the two cell types [77]. Two days after a 14-day co-culture, there were few responses to osteogenesis or angiogenesis. However, dynamic co-culture under stress led to increased bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) gene production in HUVECs, indicating the development of human bone marrow mesenchymal stem cells (hMSCs) before osteogenesis and angiogenesis.

However, Paduano et al. compared the hydrogel extracted from collagen and demineralized bovine bone with dental pulp stem cells [78]. It was found that collagen had did not significantly affect the differentiation ability of dental pulp stem cells. Although colla-



Scheme 2. Mechanism of collagen on MSCs and osteoclasts.

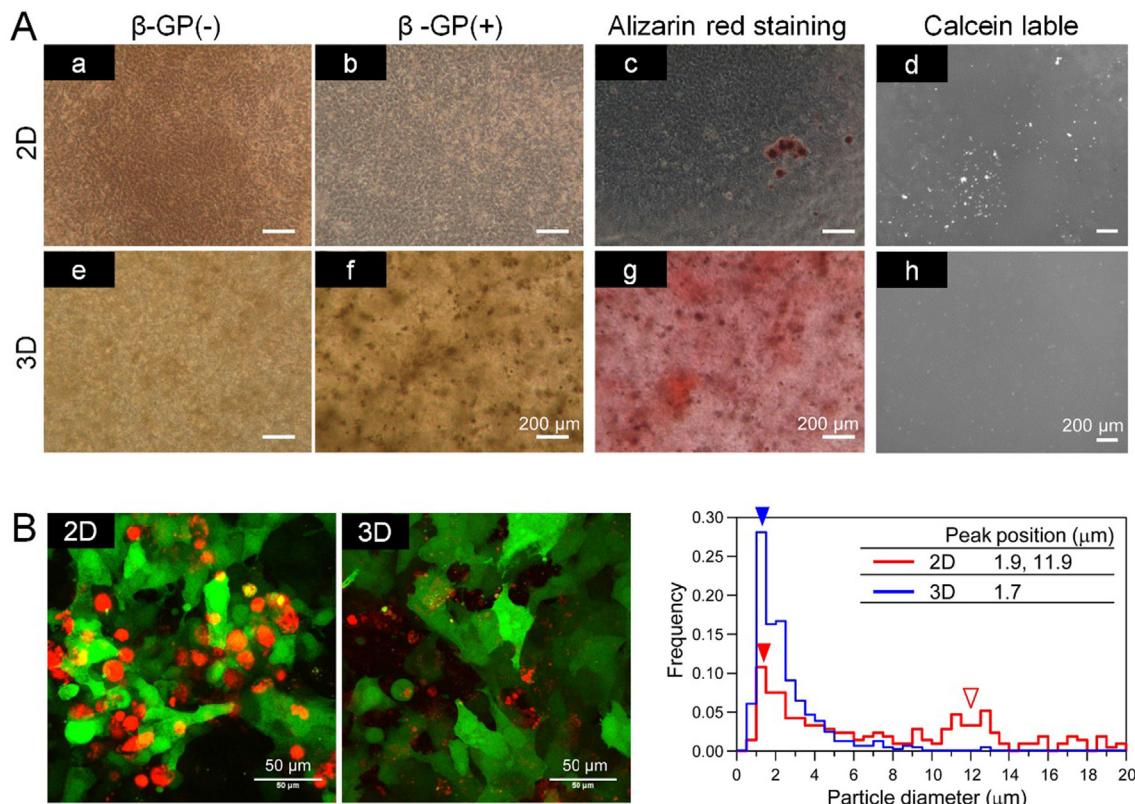


Fig. 1. (A) Phase contrast microscopy images of mineralization of HOS cells. (B) Morphological and size distribution analysis of mineralized matrices of HOS cells (Green color shows EYFP fluorescence of HOS-EYFP and red color shows xylene orange fluorescence). Adapted from a previous study, with permission [72].

gen has good biocompatibility, its osteogenic ability is still limited compared with more advanced materials. We are hopeful that transforming collagen into a more suitable material will increase its use in the future.

4.1.2. Collagen-based hydrogel synthesized with organic materials
4.1.2.1. Collagen-based hydrogel synthesized with chitosan. Low mechanical strength and high degradation rate limit the clinical application of collagen hydrogel. Chitosan is a cationic linear

polysaccharide, similar to glycosaminoglycan in the ECM, which can promote wound healing and osteoblast differentiation, and has been used to provide antibiotics to treat infections in avascular or necrotic tissues [79,80]. Additionally, chitosan is also used to promote the solubility of highly hydrophobic drugs. Chitosan/collagen composite hydrogels have been demonstrated to be biocompatible, bone conduction, and promote bone formation [81]. The crosslinking strength of both compounds is usually controlled by chemical crosslinking agents such as glutaraldehyde and epichlorohydrin to maintain mechanical strength and physical stability [82].

4.1.2.2. Collagen-based hydrogel synthesized with hyaluronic acid (Ha). Ha, the simplest glycosaminoglycan, is found in almost all mammalian tissues. It is also the main polysaccharide of the ECM, located on the surface and inside of cells [83]. Ha (a hydrogel because of its water absorption capacity) is biocompatible, biodegradable, and non-toxic [84]. Moreover, Ha shows an affinity for osteoblasts and chondrocytes, so it is often used to prepare hydrogels. Gilarska et al. used genipin as a crosslinking agent to prepare collagen/chitosan/Ha hydrogels (ColChHa) [85]. When the maximum G' of ColCh hydrogel is compared with the maximum G' of the ColChHa system, the addition of Ha affects the storage modulus, and its effect depends on Ha concentration. When Ha is stable, the amount of genipin directly determines the mechanical properties of the hydrogel.

4.1.2.3. Collagen-based hydrogel synthesized with alginate. Alginate is an excellent scaffold-forming material, which can be used to treat organ loss or failure. Alginate is composed of guluronic acid and mannuronic acid, and it is biocompatible, nontoxic, nonimmunogenic, and biodegradable [86]. Jin et al. prepared alginate/collagen gels to preserve cartilage phenotype and prevent cartilage dedifferentiation [87]. The results showed that cells proliferated over time in all gels, and the highest proliferation was achieved in the composite gel. The expression of cartilage-generating genes (including SOX9, COLII, and aggrecan) was significantly upregulated in the mixed gel. However, chondrocyte dedifferentiation markers, COLI and ALP, were also expressed significantly in the collagen gel. This reflects the importance of the gel matrix environment for the growth and preservation of chondrocyte phenotype and inhibition of dedifferentiation (Fig. 2).

4.1.3. Collagen-based hydrogel synthesized with inorganic materials

4.1.3.1. Collagen-based hydrogel synthesized with metal. The bone contact of metal-loaded implants depends on the formation of new bone tissue on the implant surface. Ideally, surface modification can promote the attachment, proliferation, and differentiation of osteoblasts. One strategy is to cover the surface with collagen fibers, the main structural protein in mammalian tissues. Titanium and its alloys, including Ti6Al4V, are commonly used as load-bearing implant materials. Type I, II, and III collagen coatings have been used to improve the adhesion and proliferation of bone-forming cells [88]. Mieszkowska et al. modified collagen hydrogels by phloroglucinol (PG) on the surface of titanium alloys as a layer [89]. The results showed that the PG-rich coating significantly reduced the expression of inflammatory markers. Furthermore, a low concentration of PG promoted the expression of osteoclast markers.

4.1.3.2. Collagen-based hydrogel synthesized with hydroxyapatite (HA). In calcium phosphate-based materials, HA can provide a strong affinity to host bone because of its similarity to the inorganic bone. HA is the main inorganic bone component because of its biocompatibility, non-inflammatory nature, and non-toxic properties. In addition to its bone conduction and biological activities, collagen-based hydrogels have been widely used to enhance their stiffness [90,91]. The mixture of collagen hydrogel and HA involves combining HA particles with collagen solution in physical form and then performing physical or chemical crosslinking [92]. These methods also have some drawbacks; that is, inorganic nanoparticles cannot be evenly dispersed in the hydrogel, and the uneven dispersion of particles and aggregates in the organic matrix may lead to poor mechanical strength [93]. Takallu et al. synthesized HA particles with uniform density distribution in collagen solution ($\text{pH} = 7.4$; temperature = 37°C) using an in situ mineralization technique [94]. The results demonstrated that HA particles occupied the blank area of the collagen network and increased the crosslinking density of the scaffolds through interactions between them, thus reducing the swelling capacity of the nanocomposite scaffolds. Moreover, HA microspheres with more fine structure have also made rapid progress.

However, the traditional preparation methods for HA microspheres mainly rely on methods involving organic solvents and surfactants, most of which are harmful to human health and the environment [95]. The hydrothermal reaction is the most commonly used method to produce coral HA scaffolds from the calcium

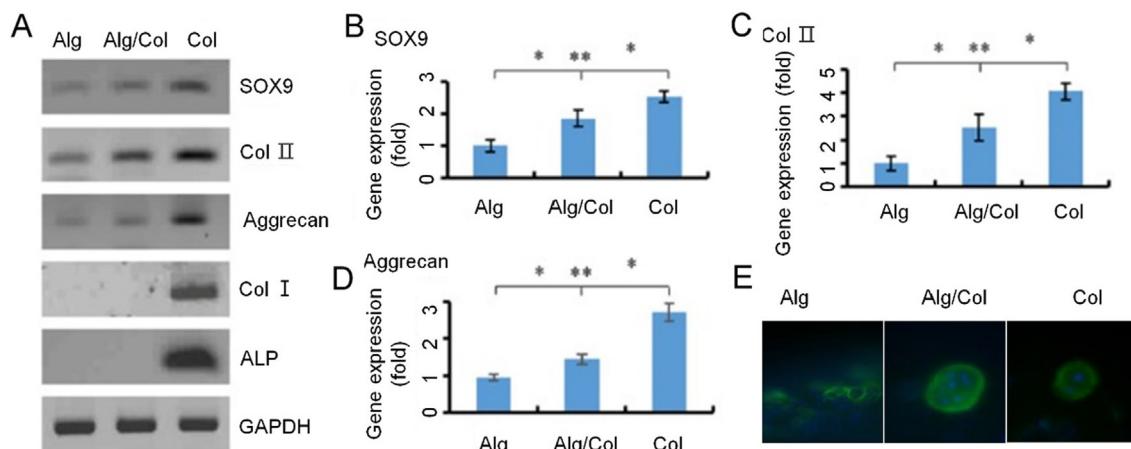


Fig. 2. (A) Western blot for chondrogenic gene expressions in various gels at day 14. (B) qPCR assay for chondrogenic gene expressions in various gels at day 14. (C) Type II collagen immunofluorescence images of the chondrocytes in various gels at day 14.

carbonate exoskeleton of marine coral calcite [96]. This strategy always involves harsh conditions, such as high temperature and high pressure, which will undoubtedly damage the biological activity of macromolecules that form a hydrogel matrix. Wei et al. developed a CaCO_3 template-based method to prepare HA spherulites in a collagen matrix and then obtained a collagen-HA microsphere composite hydrogel under mild conditions [97]. After phase transformation in the phosphate environment, CaCO_3 nanoparticles were replaced entirely by lamellar or needle-like HA nanocrystals, forming porous HA microspheres with a "cauliflower" structure or assembled into clusters of collagen fibers. The apatite microspheres derived from the CaCO_3 template showed typical cauliflower-like morphology. This kind of carbonate apatite with poor crystallinity is of great significance for the reabsorption and remodeling processes in tissues.

Soluble Ca^{2+} and PO_4^{3-} were added to cell culture medium, and then osteopontin (OPN) extracted from milk was used as nucleation inhibitor to promote protein induced collagen mineralization [98]. Gene expression analysis showed that the cells in mineralized gels had similar or higher mRNA expression of runt-related transcription factor 2 (Runx2), osteocalcin (OCN), and Podoplanin (PDPN) compare with those encapsulated in non-mineralized gels. Protein quantitation showed that the mineralized collagen gel induced a higher ratio of RANK-L: OPG (receptor activator of nuclear factor- κ B ligand: osteoprotegerin) compared with unmineralized gel or osteogenic induction medium, indicating that the material could regulate the communication between osteoblasts and osteoclast precursors.

4.1.3.3. Collagen-based hydrogel synthesized with bioactive glass. Bioactive glass (BG) is a bone-inducing, biocompatible and biodegradable material first developed by Larry Hench and his colleagues in 1969. Bioactive glass is usually made from silica and other oxides, such as calcium, phosphorus, and sodium oxides. After implantation, a HA layer will be formed on BG's surface, forming strong binding between bone and soft tissue [99]. After that, growth factors bind easier to the apatite layer and promote cell adhesion. Subsequently, as a result of HA stimulation, BMSCs differentiate into osteoblasts and induce bone formation. Various techniques have been used to fabricate collagen/BG composite scaffolds, such as freeze-drying, surface coating by immersion in BG suspensions, and collagen/BG solution transfer onto the scaffold surface [100,101]. Although these methods produce highly porous 3D scaffolds, the need to extend the processing steps limits their ease of delivery.

To solve this problem, Miri et al. developed an injectable dense collagen (IDC)-BG composite hydrogel using the gel aspiration-ejection (GAE) technique [102]. Concentration by ultrasonication to form BG-DMEM solution with a predefined mass ratio. Type I collagen from bovine dermis was added into BG-DMEM solution at a ratio of 4:1, and the final collagen concentration was 4.8 mg/ml. After NaOH neutralization, a standardized IDC processing method was established using 48-well culture plates to ensure the reproducible production of sterile IDC gel using GAE. The results showed that the addition of BG enhanced mineralization, neovascularization, and cell infiltration into the scaffolds, and there was evidence that granulation tissue in IDC-BG was remodeled into woven bone-like tissue 21 days after injection. Second harmonic generation (SHG) imaging of implanted scaffolds showed that collagen fibrils were remodeled through cell infiltration and mineralization over time (Fig. 3).

4.1.4. Collagen-based hydrogel synthesized with organic and inorganic materials

In recent years, organic and inorganic scaffolds have been widely used in bone tissue engineering. Glass based on nano- SiO_2

(n SiO_2) can induce apatite crystallization, promote cell adhesion and collagen formation, which shows the superiority of n SiO_2 in the surface reaction of scaffolds. Khatami et al. have shown that the introduction of collagen and hydrogel leads to a decrease in pore size of the alginate/n SiO_2 hydrogel [103]. Unlike hydrogel, the introduction of collagen reduced the swelling rate of alginate/n SiO_2 hydrogel. The addition of collagen and hydrogel improved the characteristics, cell proliferation, and differentiation potential of alginate/n SiO_2 hydrogel microcapsules. Nano-sized bioactive ceramic particles have some advantages over micron-sized particles because they have a larger specific surface area and can form a closer interface with polymer matrix in composite materials [104].

Because bone tissue is a multi-layered structure, multi-layer scaffolds for bone regeneration have been extensively studied. These include bioactive and hierarchical ceramic hydrogels, freeze-dried gradient hydrogels, and gelatin-based nano-sodium phosphite-gradient three-layer porous scaffolds [105,106]. Compared with single or double-layer scaffolds, the multilayer structure combines all cartilage layers, calcified cartilage, and bone. Korpayev et al. prepared each specific layer in osteochondral tissue by combining natural chitosan, collagen (type I and II), and HA in biomimetic compositions [107]. The final layered scaffolds showed a compact structure, and no separation was observed between the layers during the fabrication of the scaffolds. MC3T3-E1 pre-osteoblasts cultured in the bone layer and ATDC5 cells cultured in calcified cartilage and cartilage layer showed high viability in the co-culture medium.

Recently, synthetic polymers have received increasing attention because of their simple processability, a wide range of sources, and strong plasticity. Fu et al. developed a three-component hydrogel composite consisting of PECE copolymer, collagen, and nano-HA [108]. A rheological analysis shows that the composite has good thermal sensitivity. In vivo, it has been shown that the biodegradable PECE/collagen/nano-HA hydrogel composite has good biocompatibility and has better performance than the self-healing process in guided bone regeneration. A summary of the abovementioned studies in this section are studied in Table 2.

4.2. Collagen-based scaffolds in bone tissue engineering

4.2.1. Raw collagen-based scaffolds

Collagen scaffolds play an essential role in bone tissue engineering because they simulate the natural bone structure very well. In addition to the scaffold components, the pore size, porosity, and adhesiveness of scaffolds also significantly affect bone regeneration. Additionally, good mechanical properties and predesigned bone structure can also increase bone regeneration to a certain extent. As we all know, the rigid scaffolds made of natural collagen cannot fulfill the in vivo mechanical strength requirements, and their effect on bone defect repair is not apparent; therefore, they are primarily used as a controlling group [109].

Jiang et al. compared the repair effect of natural collagen (Col) and denatured collagen (DCol) on the cartilage [110]. Using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as the crosslinking agent, 10 ml of Col (8 mg/ml) and DCol (16 mg/ml) solutions were mixed with EDC (2 mg/ml) and then dried to make scaffolds. The results showed that compared with denatured collagen scaffolds, the natural collagen scaffolds with triple helix could promote the adhesion, proliferation, and redifferentiation of chondrocytes and the regeneration of cartilage defects. Micro-CT evaluation showed that natural collagen scaffolds induced subchondral bone regeneration (Fig. 4). Natural collagen may be more suitable for repairing cartilage defects than denatured collagen, and denatured collagen is not a good choice for cartilage engineering applications based on collagen. In another study, researchers used HA

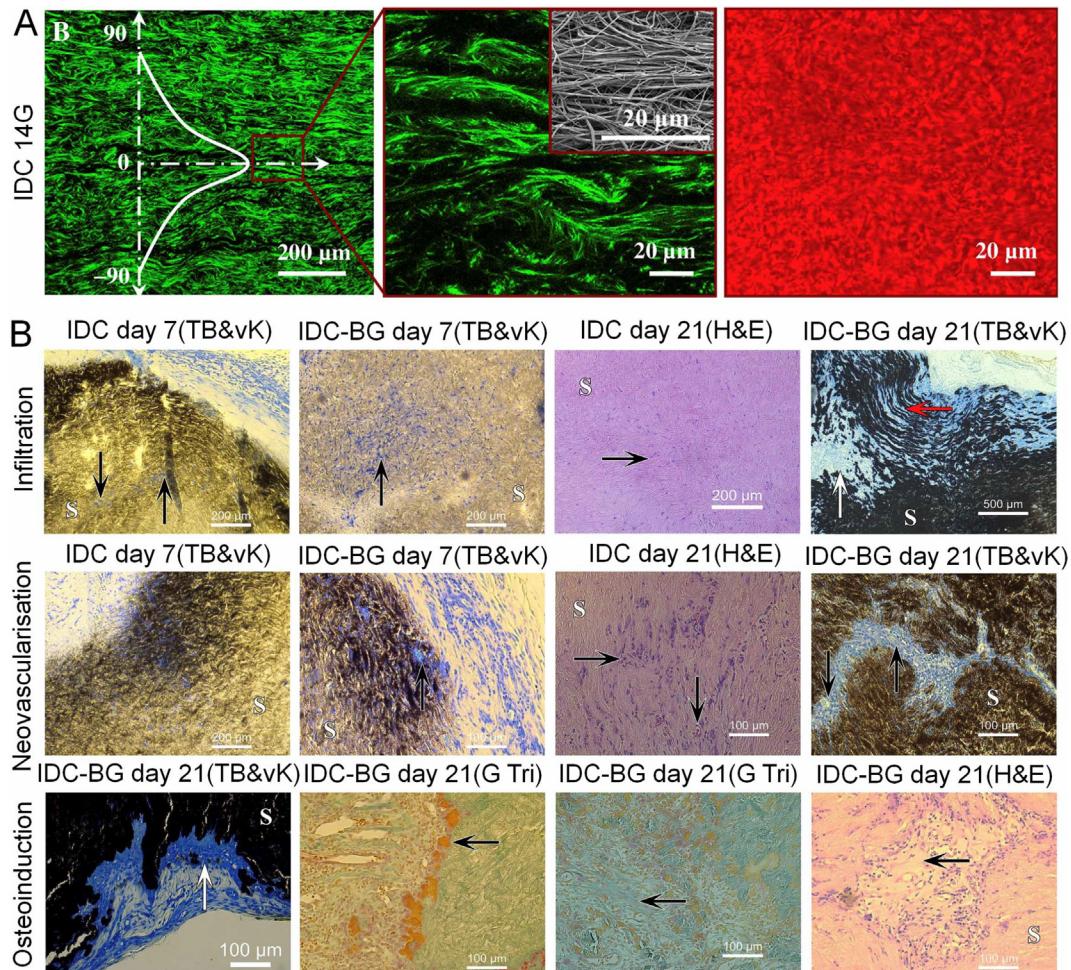


Fig. 3. (A) SHG and Dodt images of collagen fibrillar orientation. (B) Cell infiltration into the scaffolds, angiogenesis around scaffolds and bone regeneration around the scaffolds. Adapted from a previous study, with permission [102].

Table 2
Summary of collagen-based hydrogel synthesized with organic and inorganic materials.

The component ratios	Mechanisms of coupling	Optimal compositions	Stability assessment	Conclusion	Reference
Alg (1% w/v) + col (0.75% w/v) + nSiO ₂ (1% w/v), Alg(1% w/v) + gel(1.25% w/v) + nSiO ₂ (1% w/v), Alg(1% w/v) + nSiO ₂ (1% w/v)	Calcium chloride crosslinking	Alg (1% w/v) + col (0.75% w/v) + nSiO ₂ (1% w/v)	Alg + col + nSiO ₂ has a noteworthy decline in swelling rate; Alg + col + nSiO ₂ has enhanced cross-linking degree and mechanical strength; Biodegradation of alg + nSiO ₂ hydrogel was reduced following the addition of col	Detection of transcription level of osteocalcin and BMP-2 showed that col had the potential to induce bone morphogenesis; Col increased MG-63 cell survival rate after 28 days.	[103]
Bottom bone layer: chitosan (2% w/v) + col I (0.5% w/v) + nHA particles (1% w/v); Intermediate calcified cartilage layer: chitosan (2.5%, w/v) + col II (1% w/v) + nHA(0.5% w/v); Top cartilage layer: chitosan (2.5% w/v) + col II (2.5%, w/v) + nHA(0.5% w/v)	Freeze-dried, Thermal gelation method		The bone layer was found to have the highest elastic modulus of 42.95 ± 4.3 kPa; The elastic moduli of calcified cartilage and cartilage hydrogel layers were calculated as 5.41 ± 0.6 and 1.49 ± 0.3 kPa, respectively; The three layered scaffold had a compressive modulus of 0.51 kPa	MC3T3-E1 preosteoblasts cultured in bone layer and ATDC5 cells cultured in calcified cartilage and cartilage layers showed high viability in the co-culture medium.	[107]
3 g freeze-dried PECE + 0.5 g Collagen + 1.5 g n-HA	Using Sn (Oct) ₂ as catalyst; cross-linked by using HMDI as linker		<30°C: low elastic modulus (G') and viscous modulus (G'') >30°C: high G' and G''	The thermal-responsive PECE/Collagen/n-HA hydrogel composite showed the good capacity of guided bone regeneration.	[108]

and collagen to control the mechanical repair of bone [111]. The results showed that the pore size and water absorption capacity

of the experimental group decreased, but the pore size became uniform and highly connected with each other. The results of the

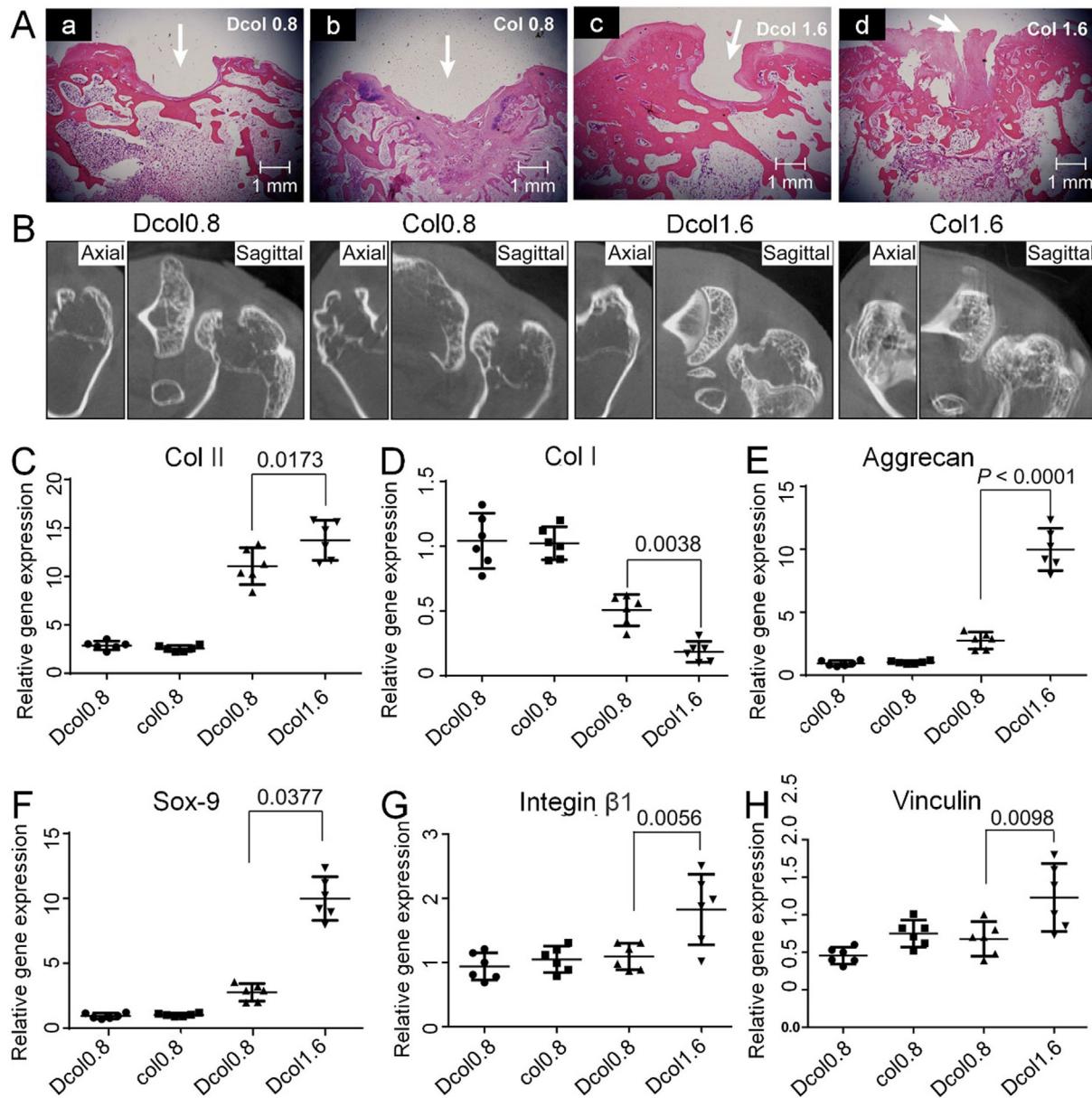


Fig. 4. (A) Histological assessment of cartilage defect repairs. (B) Micro-CT assessment of subchondral bone repair. (C-H) Expression of cartilage genes and adhesion molecules of chondrocytes cultured in scaffolds for two weeks. The Col and DCol (8 and 16 mg/mL) scaffolds were labeled Col0.8, Col1.6, DCol0.8, and DCol1.6 (Col = natural collagen, DCol = denatured collagen). Adapted from a previous study, with permission [110].

subsequent cell activity test and ALP activity test were as expected, and the control group with collagen alone was not as effective as the other two groups.

4.2.2. Collagen-based scaffolds synthesized with organic materials

4.2.2.1. Collagen-based scaffolds synthesized with chitosan. The strength of collagen scaffolds is low and cannot bear the pressure of bone tissue; however, collagen can increase cell adhesion *in vivo*. Chitosan monomers provide adjustable biomechanical properties based on their degree of chemical crosslinking, but this crosslinking process impairs cell adhesion. Kafi et al. developed collagen/chitosan composite scaffolds to improve mechanical strength and increase hMSC adhesion and proliferation [112]. The results showed that the composite scaffolds exhibited enhanced hMSC adhesion and proliferation because of their well-distributed interconnected porous structure and increased cell adhesion. There are no interconnected collagen scaffolds in the

porous structure, which shows the best growth and proliferation of hMSC on the surface, and cell distribution in the whole scaffold is poor. Similarly, the highest levels of osteoid differentiation in hMSC were observed using the chitosan/collagen composite scaffolds.

4.2.2.2. Collagen-based scaffolds synthesized with polycaprolactone (PCL). PCL is considered a candidate material for local bone defect repair because of its favorable properties, such as biocompatibility, biodegradability, non-toxicity, low degradation rate, and good mechanical properties. However, PCL's surface is hydrophobic and does not promote cell adhesion and proliferation [113]. Therefore, the combination of PCL and natural polymer can enhance the hydrophilicity, substrate softness, and biological characteristics of cell adhesion and proliferation. Nguyen et al. used abalone digestive tract protein combined with 3D printed PCL/collagen scaffolds [114]. Compared with other groups, the results showed that the

mixed group had better hardness and increased cell proliferation and differentiation in bone defect repair.

4.2.2.3. Collagen-based scaffolds synthesized with silk fibroin. Silk fibroin is an essential biomaterial with good biocompatibility, excellent mechanical properties, low inflammatory reaction, and processing versatility. Because of its excellent mechanical properties, silk is widely used in the biomedical field [115]. However, to be used in tissue engineering, the regeneration process is inevitable, which completely changes the properties of silk, especially its excellent mechanical properties. To maintain its mechanical strength, Hu et al. prepared silk/recombination human-like collagen composite scaffolds by freeze-drying methods [116]. The results showed that the compressive strength and modulus of the composite scaffolds reached 662 kPa and 7.8 MPa, respectively. Meanwhile, cell growth in the hybrid scaffolds was better than that in the pure fibrin scaffolds.

4.2.3. Collagen-based scaffolds synthesized with inorganic materials

4.2.3.1. Collagen-based scaffolds synthesized with mineral. Mineralized collagen (MC) is a biomimetic compound of collagen and hydroxyapatite, which simulates the chemical composition, microstructure, and scaffold of natural bone matrix. During MC preparation, the biomimetic mineralization process in vitro simulates the formation of a natural skeleton and is used to prepare biomimetic MC [117]. As an organic component of MC, type I collagen fibers form ordered arrangements and provide nucleation sites. Therefore, MC is closer to natural bone tissue than inorganic biomaterials in composition and microstructure. Simultaneously, MC induces the chemotaxis and differentiation of mesenchymal stem cells (MSCs) into osteoblasts and produces the natural mineralized matrix and collagen, promoting new bone tissue growth.

Studies have confirmed that the degree of osteogenic differentiation of osteoblasts is induced by biomaterials containing biomimetic collagen [118]. In a study of periodontal ligament stem cells, the mineralized collagen group showed increased expression of osteopontin, type I collagen, and BMP-2 after 7 and 14 days of culture on the fibroblast collagen [119]. The ALP activity of umbilical cord MSCs cultured on the scaffolds made of biomimetic collagen was similar to that of cells cultured in an osteogenic differentiation medium established on tissue culture plastic. The scaffolds were implanted into a rabbit femoral defect model and showed almost complete healing after 12 weeks [120]. Brendan et al. prepared collagen fibers from the decellularized tendon by frozen section and then made mineralized collagen by alternately soaking during the mineralization process [121]. With the increase in soaking cycles, the content of minerals increased. The results showed that the mechanical properties of single collagen fibrils were similar to those of natural mineralized collagen fibers, and the chemical and structural characteristics of mineralized collagen fibers with natural bone induction characteristics were also observed.

4.2.3.2. Collagen-based scaffolds synthesized with metal ion. In recent years, various metal ions have attracted many researchers for their unique role in bone regeneration. Strontium (Sr) is an essential trace element for the human body that is beneficial for bone metabolism. Therefore, the bioactive substance-releasing Sr is particularly suitable for osteoporosis patients because it can stimulate the proliferation and differentiation of osteoblasts and destroy the activity of osteoclasts [122]. Similar to Ca^{2+} , Sr^{2+} is a divalent cation, which can be incorporated into mineralized collagen to synthesize materials with improved bioactivity [123]. Qi et al. prepared type I collagen/HA strontium nanocomposites by non-classical biomimetic mineralization [124]. Polyacrylic acid was used as a biomimetic mineralizing guide agent, and nano-SrHA

mineralized collagen fibrils embedded in the gap of nanofibers were obtained. The morphology, nanostructure, and characteristics of cSrHA are similar to those of natural hard tissue and calcium mineralized HA collagen, indicating its potential value as a bone engineering biological functional material.

In another study, researchers combined magnesium ions (Mg^{2+}) with collagen/HA composition to form a new complex [125]. Bovine type I collagen was dissolved in acetate buffer (pH 3.5), then H_3PO_4 was added, homogenized with a mechanical stirrer, and then Ca(OH)_2 and MgCl_2 were dropped in deionized water. The scaffolds were prepared by wet crosslinking in 1,4-butanediol diglycidyl ether aqueous solution at 4 °C and washed with deionized water three times. The results showed that compared with the control group, the experimental group's cell morphology and osteogenic differentiation ability were significantly improved. Histological staining at six weeks demonstrated the osteoinductive properties of the complex (Fig. 5).

Copper is effective against fungi, Gram-positive and Gram-negative bacteria [126]. Furthermore, copper ions can make collagen mature through lysyl oxidase crosslinking and enhance bone formation by inducing osteogenic differentiation of MSCs. The effect of copper on vascular stimulation comes from its ability to stimulate bone marrow-derived stem cells to produce VEGF [127]. Copper-doped bioactive glass combined with porous 3D collagen scaffolds (CuBG-CS) can induce bone formation and angiogenesis while limiting infection [128]. The scaffolds were fabricated by freeze-drying a co-suspension of collagen and bioactive glass particles (with or without copper doping, referred to as CuBG and BG, respectively) at a range of different concentrations (collagen only, 20%, 100%, and 300% BG or CuBG wt/wt bioactive glass to collagen). CuBG (300%) scaffolds resulted in a significantly increased antibacterial activity versus all other groups, inhibiting *S. aureus* growth by up to 66%. A time-kill assay showed that 100% CuBG-CS also significantly delayed the growth of *S. aureus* at 7 h. Although some reduction was seen in cell number at later time points in osteogenic medium for CuBG-CS (300%), it was not significantly different compared with collagen controls at any time point. Except for the CuBG-CS (300%), there is an increase in cell number on all scaffolds containing bioactive glass. CuBG (100%) scaffolds show significantly enhanced angiogenesis compared with the collagen control both qualitatively and when quantified using image analysis software. (Fig. 6). A summary of the abovementioned studies in this section are presented in Table 3.

4.2.3.3. Collagen-based scaffolds synthesized with hydroxyapatite. The synthesis of mineralized collagen from hydroxyapatite is a common method for researchers to simulate bone tissue. Collagen/HA scaffold has certain porosity and compression modulus, and cells can survive in it [129]. However, when other components are involved, the performance of the scaffold is improved in all aspects. Li et al. proposed a multi *in situ* synthesis method to synthesize carbon nanotube/collagen/HA (CNT/COL/HA) composites with good biological and mechanical properties [130]. The multi *in situ* synthesis process ensures excellent CNT dispersion in the HA matrix, and collagen also ensures the close chemical bond between CNT reinforced material and HA matrix. The flexural strength and fracture toughness of the CNT/COL/HA composite (3 wt% CNT) was increased by 74.2% and 274.6%, respectively. This increase was much higher than that observed for the CNT/HA composite. This material can solve the defects of cytotoxicity and HA strength resulting from CNT diffusion [131].

4.2.4. Collagen-based scaffolds synthesized with organic and inorganic materials

Osteochondral defects, including articular cartilage and subchondral bone defects, are common joint problems [132]. Because

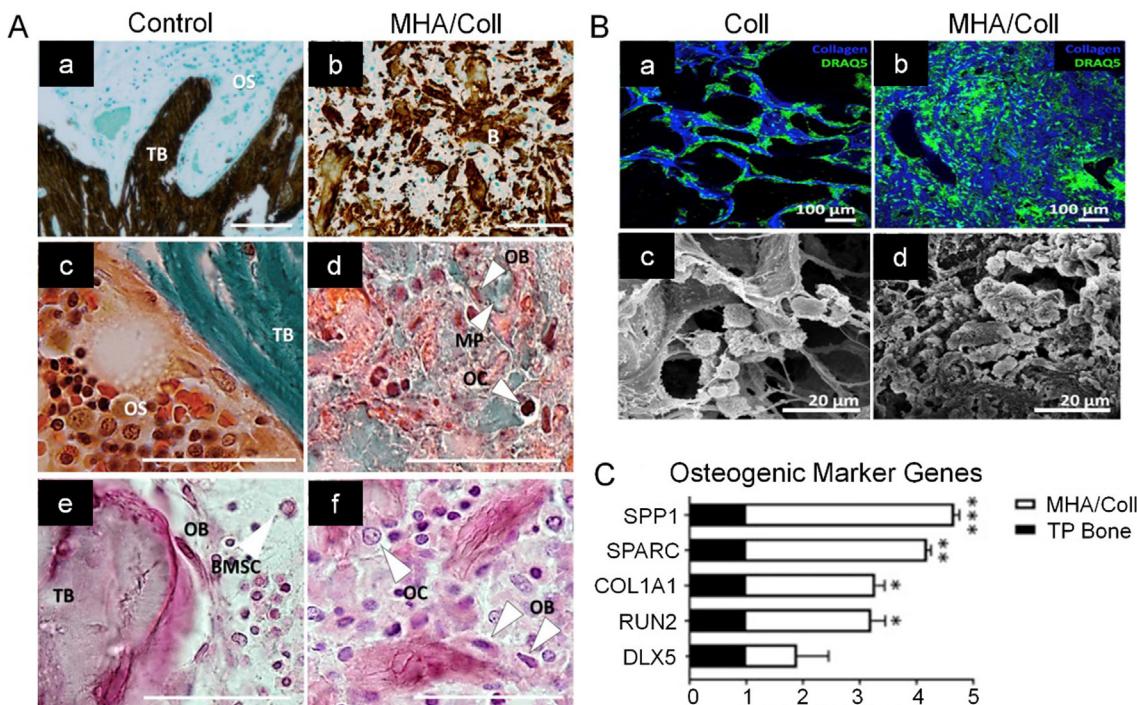


Fig. 5. (A) Von Kossa (a, b), Goldner's Trichrome (c, d), and hematoxylin-eosin (e, f) staining of non-deminerlized vertebral trabecular bone and magnesium-doped hydroxyapatite (MHA)/Coll specimens. (B) Confocal laser microscopy and SEM images of hBM-MSCs (green) cultured on Coll and MHA/Coll at three weeks. (C) Relative-fold expression of osteogenic marker genes detected for MHA/Coll group at six weeks after implantation in the orthotopic model in rabbit, compared with naive spinal bone. Adapted from a previous study, with permission [125].

of the complex anatomical structure at the interface between subchondral bone and cartilage, the graft design becomes complicated. Zhou et al. prepared double-layered, collagen-based composite scaffolds with small holes (about 128 μm) in the top layer and large pores (about 326 μm) in the bottom layer to simulate the morphology of cartilage and bone tissue [133]. Furthermore, the top layer was modified with chondroitin sulfate, and the bottom layer was modified with HA to further simulate the chemical composition of cartilage and bone tissue, respectively. The results showed that the double-layer scaffolds provided a basic microenvironment for cartilage formation and osteogenic differentiation of BMSCs, and eventually led to the simultaneous repair of cartilage and subchondral bone in the rabbit model.

Another study prepared Col I/PCl/ATP (CPA) scaffolds by salt leaching methods, which effectively made up for the shortcomings of a single material, such as high brittleness, rapid degradation, and lack of cheap bone-promoting materials. It was found that sodium chloride in both CP and CPA scaffolds produced macropores (about 200–500 μm), which was very important for cell growth and nutrient transport. CPA played a positive role in the subsequent tissue staining and mRNA content determination.

5. Collagen-based materials loaded with bioactive substances in bone tissue engineering

Collagen-based materials carrying bioactive substances are called carriers. Hydrogel carriers carrying bioactive substances usually need to adhere to other hard materials before they can become an in vivo graft. Because the scaffold itself has certain compressive strength, it can directly carry bioactive substances, and can also carry bioactive substances by coating hydrogels. Bioactive materials represent the materials that play a positive role in the integration of bone tissue engineering. The promotion effect of sin-

gle collagen-based materials on bone integration and bone regeneration is limited, so the participation of bioactive substances (such as growth factors, drugs and cells) will increase the bone integration effect of collagen-based materials. The carriers are collagen-based materials carrying bioactive substances. Hydrogel carriers carrying bioactive substances usually need to adhere to other hard materials before they can become an in vivo graft. Because the scaffold itself has certain compressive strength, it can directly carry bioactive substances, and can also carry bioactive substances by coating hydrogel.

5.1. Collagen-based hydrogel carriers

Since the bionic strategy has been widely accepted, various factors have been proposed as requirements for hydrogel [134]. Bionics involves assessing the morphology and chemical composition of scaffolds and the molecular interactions in the microenvironment. Good delivery system can release bioactive substances slowly to target cells and make them act on cells for a long time. In this case, many local delivery systems have been developed that exhibit controlled and sustained release.

5.1.1. Collagen-based hydrogel loaded with growth factors for osteogenesis and vascularization

Recently, the application of BMP has become an effective treatment in bone reconstruction surgery. BMP induces bone formation by regulating the recruitment and differentiation of osteoprogenitor cells [135]. Of the many BMPs, BMP-2 and BMP-7 are now in clinical use. However, the clinical application of BMP is limited by its short half-life and quick loss of biological activity [136]. Moreover, BMP-2 is easily lost when applied locally; therefore, it usually requires large doses, which results in high costs. The osteogenic efficacy, bioactivity, and optimal dose of BMP-2 depend on its delivery carrier [137,138]. Compared with the same amount of

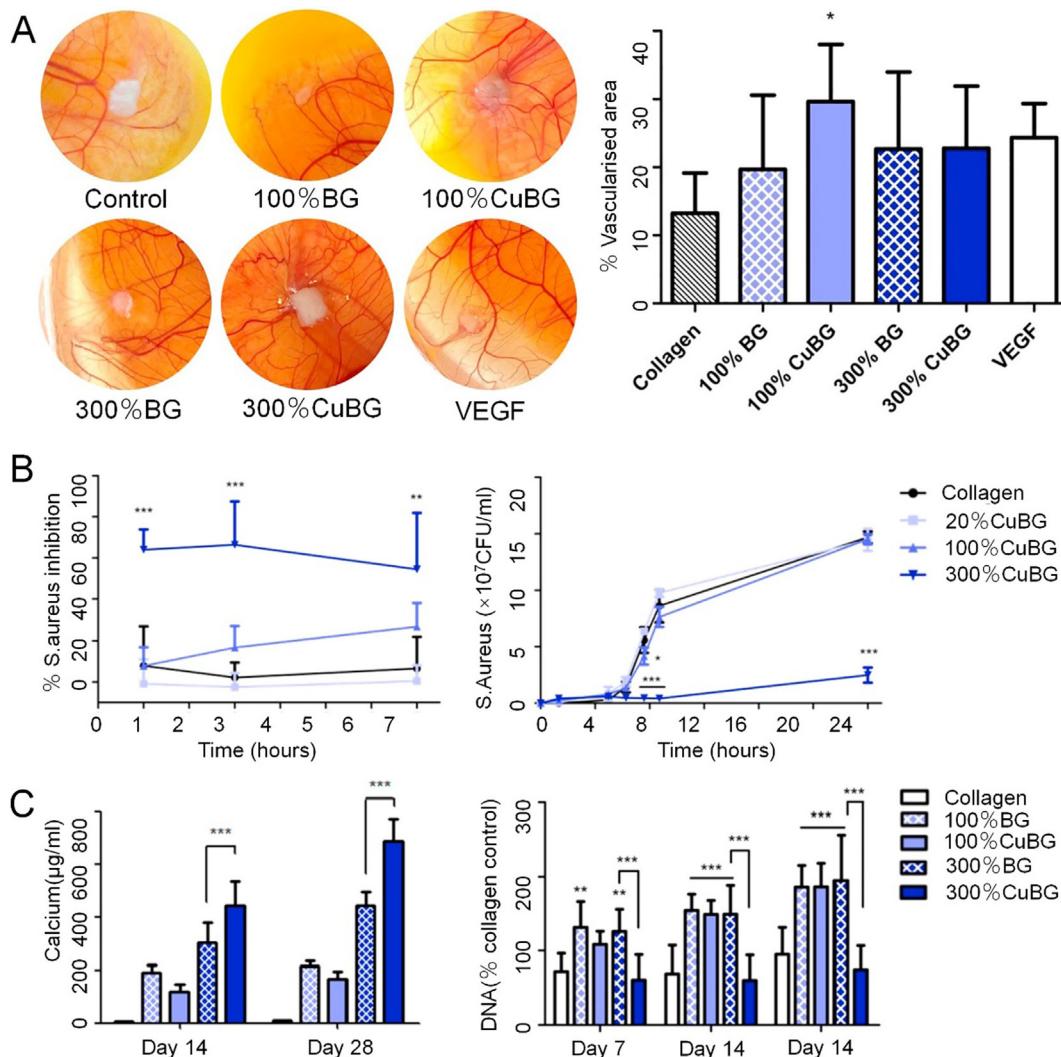


Fig. 6. (A) Effect of bioactive glass scaffolds on angiogenesis in a chick embryo ex ovo model. (B) Effect of bioactive glass addition on the antibacterial activity of scaffolds. (C) The ability of copper-doped bioactive glass scaffolds to enhance osteogenesis. Adapted from a previous study, with permission [128].

Table 3
Summary of collagen-based hydrogel synthesized with metal ions.

The component	Mechanisms of coupling	Metal ion	Stability assessment	Conclusion	Reference
Collagen, Sr, HA	Biomimetic mineralization	Sr	A cross-sectional view of mineralized collagen fibrils showed platelet nanocrystals throughout the full thickness of the collagen fibril.	Collagen/Sr/HA displayed nanostructures and characteristics similar to those of natural hard tissue, indicating its potential value as a biofunctional material for bone engineering.	[124]
Bovine Col I, HA, Mg	Bone biominerlization	Mg	The porosity of Mg/HA/Col I was assessed at 70%; Overall Young's modulus close to 6 MPa.	Mg/HA/Col I, based on magnesium-substituted hydroxyapatite and type I collagen, synthesized through a bioinspired mineralization process of type I collagen; Its has ability to induce, orchestrate, and harmonize the osteogenic differentiation of hBM-MSCs.	[125]
SiO ₂ , CaO, P ₂ O ₅ , CuO	A sol-gel process	Cu	Increasing bioactive glass concentration increased scaffold compressive modulus with a linearly increasing trend.	The incorporation of CuBG enhanced osteogenesis and angiogenesis in a dose-dependent manner; CuBG addition was shown to result in antibacterial activity, demonstrating increased toxicity against S. aureus.	[128]

short-term delivery vector, the BMP-2 long-term delivery vector can enhance the osteogenic effects of protein. The long-term delivery vector can continuously release BMP-2 while retaining its biological activity [139]. Yang et al. used collagen for long-term BMP release, and they showed that BMP encapsulated in collagen could be continuously released for up to 30 days [140]. The subsequent

ALP activity test also showed that the released BMP retained its biological activity.

The use of commercial collagen to release BMP-2 usually leads to very low utilization of BMP-2 [141]. Therefore, the design of an ideal hydrogel/matrix capable of loading BMP-2 at low doses and sustained release is essential for its successful therapeutic applica-

tion to enhance osteogenesis. The full-length recombinant collagen was designed to achieve high mechanical strength, stable release rate, and low immunogenicity [9]. The failure and adverse side effects of BMP-2 in bone repair mainly result from high dose administration and quick delivery. Therefore, significant efforts have been made to obtain the “ideal” hydrogel/matrix, which has a low dose BMP-2 load and sustained release. On this basis, Chen et al. used biological enzymes to strengthen the crosslinking of recombinant human-like collagen (HLC) to achieve an effective and safe BMP dose [142]. The crosslinked HLC hydrogel was first transformed into an HLC sponge by freeze-drying. After absorbing BMP-2, the HLC sponge became an HLC-BMP hydrogel. The results showed that the morphology of BMSCs wrapped in collagen was good, and the pseudopodia structure was extended. According to the amount of amplified DNA, the BMSC osteogenic genes were higher than those of the control group. The same conclusion was also obtained by different staining results (Fig. 7).

Huang et al. developed a collagen/chitosan/HA injectable hydrogel for promoting orthotopic bone formation [143]. For specific applications, the thermal methods may be advantageous because they do not require organic solvents, copolymers, or external application triggers of gelation. The chitosan/beta-glycerin phosphate preparation will undergo a thermosensitive sol-gel transition under specific temperatures [144]. These preparations

have physiological pH values and can be kept liquid below room temperature to encapsulate living cells.

BMP-2 is the initial member of the transforming growth factor-beta (TGF- β) superfamily expressed during fracture healing, while BMP-7 is usually expressed 2 weeks after fracture [145]. Yilgor et al. found that the sequential strategy of delivering BMP-2 first and then BMP-7 promoted the proliferation and differentiation of mesenchymal cells into osteoblasts compared with simultaneous delivery [146]. Based on the results of slow delivery of collagen by Jo et al. [147], it was shown that BMP-7 was slowly released from the membrane, while BMP-2 was rapidly released. Compared with a single delivery of BMP-2 and BMP-7, sequential delivery of BMP-2 and BMP-7 using a heparinized collagen membrane significantly induced new bone formation.

Soluble factors such as growth factors that regulate bone regeneration are potential candidates for enhanced osteogenic differentiation. For example, basic fibroblast growth factor (bFGF), as a potential candidate for enhanced osteogenic differentiation, promotes increased proliferation of osteoblasts in monolayer cells and organ culture models and leads to enhanced bone formation [148,149]. Oh et al. reported the effect of bFGF on the osteogenic differentiation of MSCs in collagen hydrogels to take advantage of 3D culture conditions that better represented the natural tissue environment *in vivo* [150]. A significant increase in osteogenic

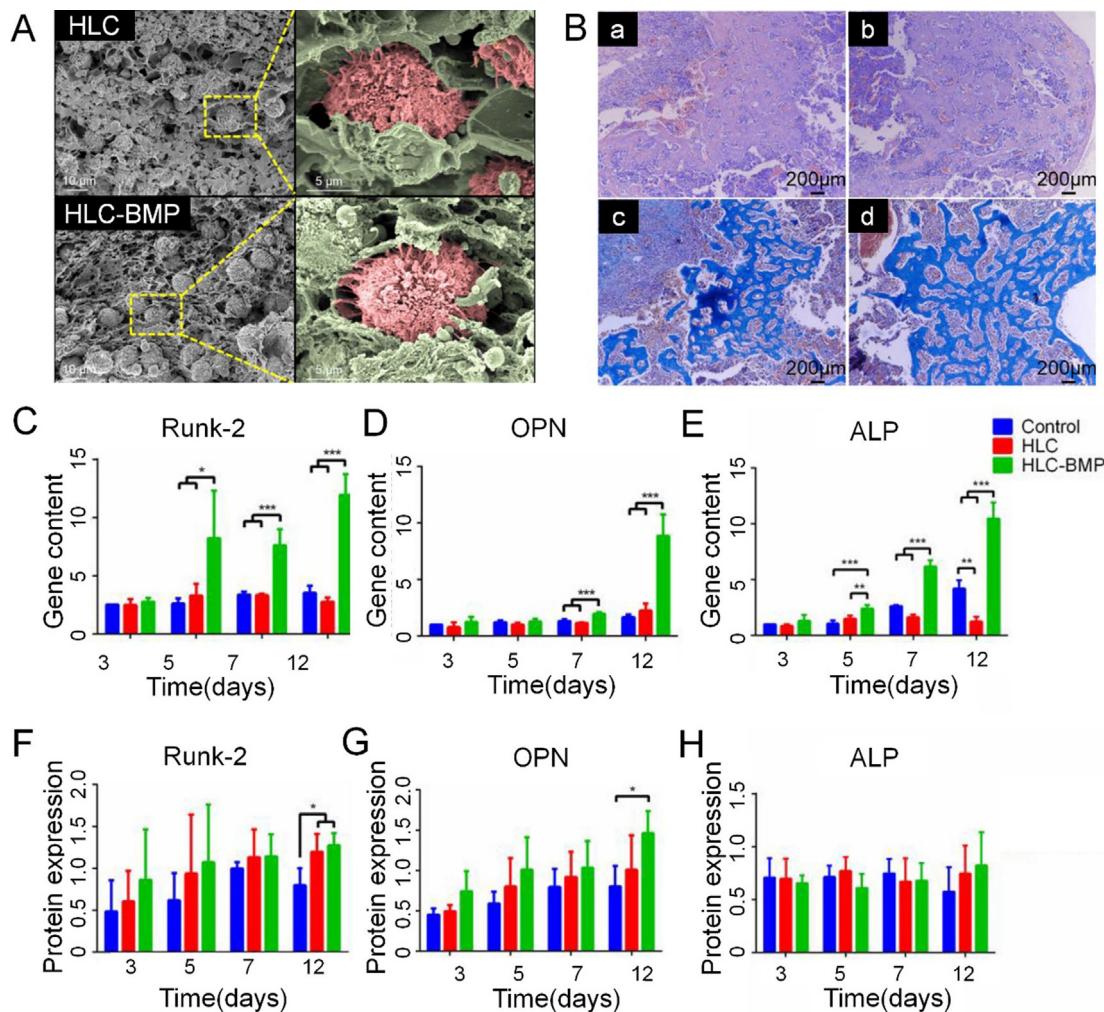


Fig. 7. (A) Collagen and BMSCs under an electron microscope. (B) H&E (a, b) and Masson's trichrome staining (MTS) stain (c, d) for HLC-BMP after implantation for 4 weeks, respectively. (C-E) Gene content of Runx-2, OPN, and ALP. (F-H) Protein relative expression of Runx-2, OPN, and ALP. Adapted from a previous study, with permission [142].

gene expression was observed on day 14 in the presence of bFGF compared with the control without bFGF. In another experiment using canine teeth, the FGF-2 wrapped in collagen hydrogel promoted periodontal defect healing and avoided the abnormal healing (such as ankylosis) resulting from BMP [151]. On the 10th day, FGF-2 was applied to the scaffolds to promote the inward growth of a large number of cells and tissues, including a large number of cells and vascular-like structures. On the fourth week, alveolar bone reconstruction was stimulated by the implantation of FGF-2 loaded scaffolds.

VEGF can enhance osteogenesis through osteoblast differentiation and the transport of precursor mesenchymal cells to mineralized areas through newly formed vessels, which may provide a useful compensatory role in bone regeneration of low-dose BMP-2 [152,153]. Furthermore, the synergistic effect of VEGF and BMP-2 may be beneficial to enhance bone regeneration, especially for the early functional load of dental implants after bone grafting. Kim et al. used collagen hydrogels mixed with BMP-2 and VEGF and compared the effects of the BMP-2 group with that of the BMP-2 and VEGF group on alveolar bone defects in canine models [154]. The total amount of new bone formation and bone mineral density in the BMP-2 group and the BMP-2 combined with VEGF group were significantly higher than those in the control group. In the group with BMP-2 and the group with BMP-2 and VEGF, the positive staining area of the von Willebrand factor in the bone defect was significantly larger than that in the control group.

5.1.2. Collagen-based hydrogel loaded with drugs for osteogenesis and vascularization

Collagen is not only coated with BMP but also widely used as a carrier of other drugs. It can maintain the biological activity of loaded drugs for extended periods. Nabavi et al. loaded tacrolimus, a macrolide antibiotic and an immunosuppressant, into collagen hydrogel [155]. The outer layer of the complex was coated with PCL/gelatin film. It reduces the production of IL-2, thus reducing the production and proliferation of T lymphocytes [156]. The results showed that the bone formation of the tacrolimus group was significantly improved compared with other groups. The bone mass analysis showed that the tacrolimus group had about 10% more bone mass than the non-tacrolimus groups. Several studies have claimed that tacrolimus can enhance osteogenic differentiation by binding to FK506-binding protein 12 and activating BMP receptors. At present, the mechanism remains unclear, but some studies claim that tacrolimus can enhance osteogenic differentiation by binding to FK506-binding protein 12 to activate the BMP receptor [157]. After tacrolimus was combined with BMP-4, the proliferation and differentiation activities of MC3T3E1, ST2, and C3H10T1/2 cells were analyzed. The results showed that tacrolimus further enhanced the osteogenic differentiation of three types of cells by enhancing ALP activity [158].

Icariin (ICA) is a type of flavonoid component isolated from the ewe herb, which has been widely studied in regenerative medicine. Many studies have shown that ICA can enhance the osteogenic differentiation of MSCs, promote matrix calcification, and inhibit osteoclast bone resorption with or without induction medium [159–161]. Simultaneously, other studies have pointed out that ICA can promote MSC chondrogenesis in the induction medium, promote the proliferation and maintenance of the chondrocyte phenotype, increase the secretion of proteoglycans and collagen matrix by chondrocytes, and inhibit the degradation of collagen and proteoglycan [162–164]. ICA was conjugated to the hyaluronic acid/collagen (Ha/Col) composite hydrogel, which inhibited the initial burst release and maintained ICA release [165].

The hydrogel constructs promoted the encapsulation of chondrocytes to secrete more proteoglycans and collagen matrix. Yang et al. used an ICA-conjugated Ha/collagen (ICA-Ha/Col) composite

hydrogel to encapsulate BMSCs, and BMSC/hydrogel constructs were cultured in a differentiation-inducing medium to stimulate cartilage formation and osteogenic differentiation of BMSCs [166]. The results showed that ICA-HA/Col hydrogel could effectively enhance the osteogenesis and chondrogenesis of BMSCs compared with HA/Col hydrogel alone.

Although some growth factor co-administration strategies reported improvements in treatment effects, others reported transient to no effects [167–170]. The capillary network plays an essential role in bone regeneration, but there is also evidence that the initial vascular system is immature and leaky [171]. A good solution is to use microvascular segments that retain the vascular support cells, which may accelerate the maturation and patency of blood vessels. Ruehle et al. implanted microvascular fragments into the body to accelerate vascular network reconstruction [172]. However, the results showed that the microvascular patch could only increase the vascular area in vitro but not in vivo after implantation.

There are many different factors that can affect drug release. Drugs are wrapped in water filled hydrogels, therefore the diffusion of water filled pores is one of the release mechanisms of the whole release phase. In addition, because collagen degrades, the degradation rate of collagen-based hydrogel will also accelerate drug release. In one study, a confocal microscope was used to monitor drug release and found that swelling can lead to sudden release [173]. Therefore, swelling property of collagen-based hydrogels is another accelerating condition for drug release.

5.1.3. Collagen-based hydrogel loaded with cells for osteogenesis and vascularization

Stem cell regenerative medicine is a relatively new field of biomedicine, which has significant value in clinical application. It aims to promote the repair and treatment of diseases through stem cell transplantation, differentiation, and tissue regeneration. Hopefully, this will change traditional disease treatment methods and allow for revolutionary alternatives for research and clinical application [174,175]. The typical two-dimensional (2D) cell culture system cannot completely mimic the *in vivo* microenvironment that naturally regulates stem cell behavior. However, the 3D structure can mimic the *in vivo* environment that directly interacts with cells [176,177]. Because of the interactions between the RGD (arginine-glycine-aspartate) sequence and stem cell integrin receptors, 3D collagen scaffolds can increase the adhesion, proliferation, and differentiation of stem cells [178]. Meanwhile, the degraded components of collagen scaffolds can be reused by cells to form new tissues [179]. Before gelation, cells are suspended in the hydrogel precursor solution, and the cells are evenly distributed in collagen colloids. Zhang et al. encapsulated BMSCs in the collagen hydrogel after photochemical crosslinking [180]. The results showed that the Col/HA hydrogel provided a more suitable microenvironment for BMSC adhesion and proliferation. In the absence of BMP-2, the collagen hydrogel increased the expression of BMSC-related osteogenic genes compared with the control group. The results of ALP activity, OCN activity, and osteogenic protein expression further showed that the Col/HA hydrogel could promote osteogenic differentiation. Moreover, the new type of alginate/collagen hydrogel with a core-shell structure is used as a carrier for cell delivery in tissue engineering, which can also effectively promote the proliferation and differentiation of MSCs [181].

Nanoscale materials have been incorporated into polymers to provide nanocomposites with unique properties (such as shear thinning). Recently, low-dimensional anisotropic nanomaterials (such as graphene oxide and nanosilicate) have been integrated into the hydrogel to achieve post-gelation [182,183]. Cellulose nanocrystals are incorporated into the covalently crosslinked

hydrogels. These hydrogels have enhanced mechanical strength, exhibit viscoelasticity resulting from reversible hydrogen bonds, and can be successfully used for cell delivery [184–186]. Unlike design for cell delivery in tissues (for example, heart and brain), injectable hydrogels for cartilage defects need to be designed to fill irregular defects. The reaction of aldehydes and amino groups to form Schiff base bonds can achieve dynamic equilibrium (occurring in aqueous solution at room temperature or physiological temperature) and give hydrogels injectable and self-healing properties [187,188]. Additionally, small-molecule drugs and biomimetic peptides have been incorporated into the injectable hydrogel, and together with MSCs, promote bone regeneration in various challenging bone defect models [189,190].

In the process of bone regeneration, the supply of oxygen and nutrients is essential. Therefore, in addition to VEGF, the interaction between endothelial and stem cells can be used to promote ossification and vascularization [191,192]. It is well known that HUVECs regulate angiogenesis, the reduction of hypoxia, and apoptotic cell death. By studying the prevascularized bone tissue complex of collagen/fibrin hydrogel-coated mesenchymal stem cell/HUVEC spheres, it was found that the synergistic effect of the two cells in 3D co-culture increased regenerated bone and angiogenesis markers [193]. The acidic collagen solution extracted from the rat tail was mixed with acidic collagen solution to make a collagen solution. The fibrin hydrogels (loaded spheres) were mixed with collagen solution in a 1:1 proportion to obtain the ideal sphere density. Group 4 showed that a prevascular network was formed in the whole hydrogel, which may provide oxygen and nutrients for the encapsulated cells. Groups 3 and 4 exhibited stronger expression of Runx-2 compared with Groups 1 and 2. Furthermore, Group 4 showed the highest fluorescence intensity of Runx-2 compared with the other groups. The hydrogel can act as a highly hydrated 3D structure to protect encapsulated cells and provide a microenvironment to increase cell adhesion and proliferation (Fig. 8).

A summary of the abovementioned studies in this section are presented in Table 4.

5.2. Collagen-based scaffolds carriers

The rough surface of the newly synthesized collagen scaffolds is favorable for loading bioactive substances (such as drugs and cells). Although a single scaffold without active ingredients can also have a therapeutic effect on bone defects, it still needs the participation of other components.

5.2.1. Collagen-based scaffolds loaded with growth factors for osteogenesis and vascularization

In the clinical treatment of bone regeneration, an excess dose of bone morphogenetic protein (BMP) is administered for the local promotion of bone defect repair. However, when BMP is released in large quantities, it may result in side effects, such as excessive bone regeneration, heterotopic bone formation, and detrimental immune responses [194,195]. Delivery of BMP-2 for up to 21 days would be suitable for effective bone formation, as BMP-2 expression was observed from day 2 to day 21 in the fracture area of mice [196]. In higher mammals, BMP may have to be present for more extended periods. The initial burst release of BMP is inevitable under normal conditions; therefore, much exploration has been carried out to realize the slow release of BMP from scaffolds. Yang et al. usedapatite coating to modify the collagen scaffolds and significantly increase the BMP-2 release time and the osteogenic effect of BMP-2 [197]. In this study, with the increase of simulated body fluid (SBF) infiltration concentration, the initial burst release of BMP encapsulated in scaffolds gradually decreased and was complete after approximately 30 days.

Linh et al. prepared collagen and BMP as modified coating scaffolds for inducing osteogenic differentiation of human adipose-derived MSCs [198]. HA scaffolds were incubated with bovine serum albumin overnight and crosslinked to form scaffolds (incubated in collagen solution overnight). BMP-2 adhered to the scaffolds after 24 h of coupling at low temperatures. The results showed that by attracting cells to the wound environment and scaffold surface, bone formation was accelerated, and critical size defects were resolved. It is also essential that the modification process does not affect the structural integrity of the HA scaffolds. Therefore, the combination of stable HA scaffold platforms and collagen coating to improve cell adhesion and BMP-2 binding to promote bone matrix differentiation can be used as a new method to treat segmental bone defects (Fig. 9).

The primary source of collagen is animal tissue, which increases the risk of infectious disease transmission (such as infectious spongiform encephalopathy), and raises concerns about purity, quality, and predictability of performance [199]. Hou et al. used atelocollagen, a natural biomaterial produced by bovine type I collagen, to replace the traditional collagen [200]. The results showed that at 7 days, sporadic cartilage formation sites, new connective tissue, and ECM deposition were observed in some areas of the absorption scaffolds. At 14 days, the scaffolds were replaced entirely by new bone tissue. These new matrix/rhBMP-2 composite scaffolds are expected to provide a potential strategy for the regeneration of periodontal defects and bone repair of dental implants using tissue engineering.

The combination of collagen and BMP also plays an essential role in periodontal injury healing. Compared with cell transplantation, it is a cheap material that can be widely used in clinical trials. In vitro and in vivo studies showed that fibroblasts grew in collagen gel. Additionally, other studies have added BMP to the surface of collagen scaffolds to improve periodontal healing [201]. In the treatment of periodontal injury, BMP acts on the surface of a periodontal defect, which may cause severe ankylosis and hinder periodontal ligament formation. The scaffolds can provide the best environment for cell migration and proliferation in the regeneration space [202]. The results showed that the periodontal ligament with cementum tissue had good reconstruction on the root surface for BMP application, but ankylosis was rarely observed. BMP-modified periodontal tissue cells can specifically inhibit the occurrence of ankylosis. Previous reports have shown that the activity of periodontal ligament cells is stimulated by the application of type I collagen [203].

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5.2.2. Collagen-based scaffolds loaded with drugs for osteogenesis and antibacterial activity

In the general treatment of fracture injury, different degrees of infection often occur because of the long exposure time of the fracture. Despite the rapid development of bone tissue engineering, the prevention of significant bone loss in non-sterile wounds remains a

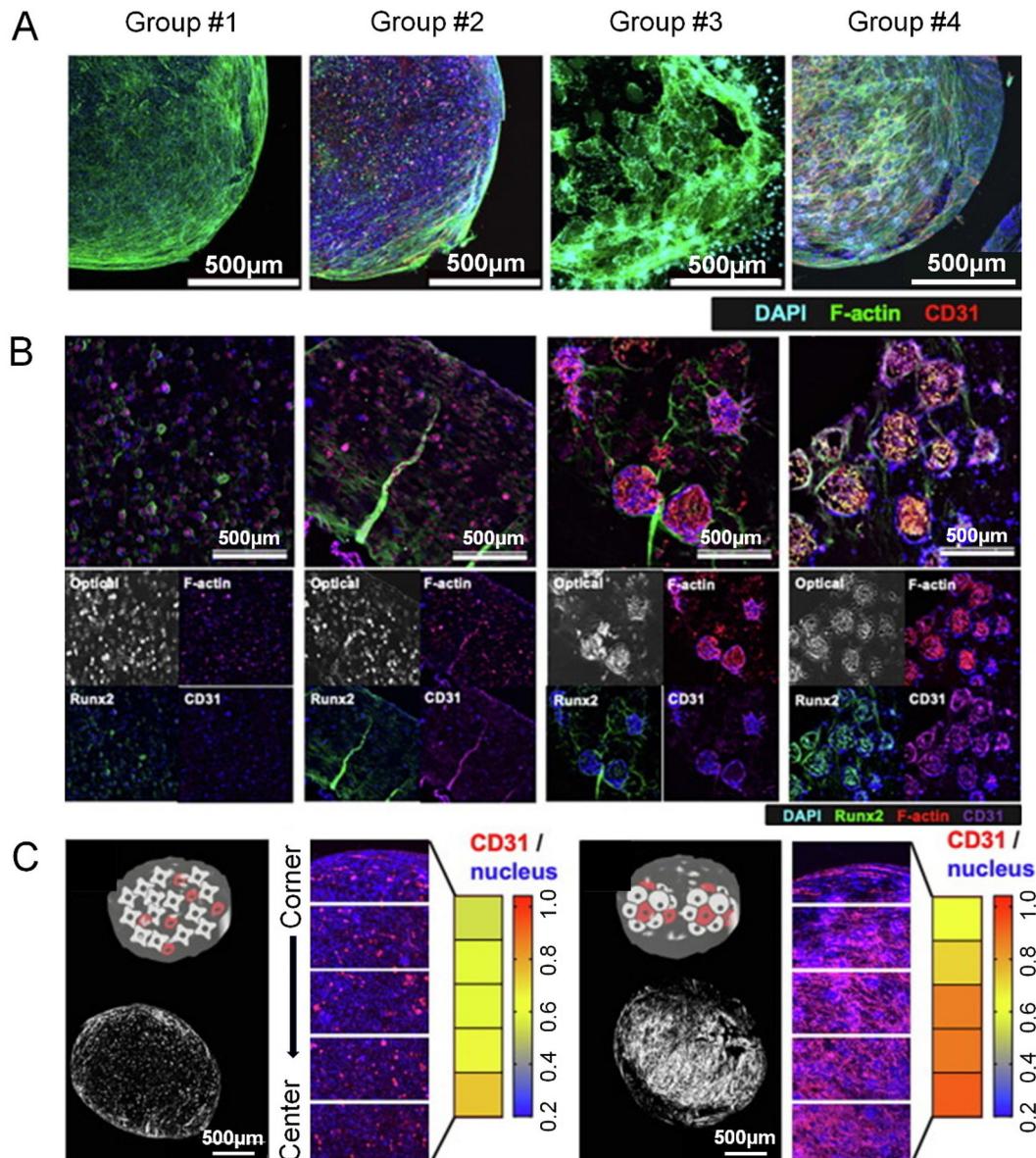


Fig. 8. (A) Formation of the actin cytoskeleton and prevascular pattern after 7 days of culture. (B) After 3 weeks of culture, osteogenic and vascular markers were stained and imaged. (C) The formation of prevascular structures was expressed by CD31/nuclear fluorescence intensity after 7 days of culture. (MSC-suspension = Group 1, MSC/HUVEC suspension = Group 2, MSC-only spheroids = Group 3, and MSC/HUVEC spheroids = Group 4). Adapted from a previous study, with permission [193].

Table 4
Summary of collagen-based hydrogel synthesized with cells.

Differentiation Direction	Cell Type	Materials	Identify Method			Reference
			Staining	RT-PCR	Others	
Osteogenesis	BMSCs	Collagen, Ha, Glycidyl methacrylate, Methacrylic anhydride	Live/dead staining, ARS and ALP staining	Runx2, ALP, OCN, Col I	NMR spectra, SEM, Western blot,	[180]
Osteogenesis	MSCs	Collagen, Alginate	Immunofluorescence staining, HE staining, Masson's trichrome staining	BSP, OPN, OCN	Dynamic mechanical analysis, DNA quantification, Trypan blue screening, Microscopic images, µCT	[181]
Osteogenesis, Angiogenesis	MSCs, HUVECs	Collagen, Fibrin	Live/dead staining, Immunofluorescence staining, Alizarin red S staining, HE staining	Runx2, ALP, OCN, Col I	Microscopic images	[193]
Chondrogenesis	MSCs	Collagen,CNCs	Live/dead staining, HE staining		FT-IR, Mechanical tests, SEM	[186]

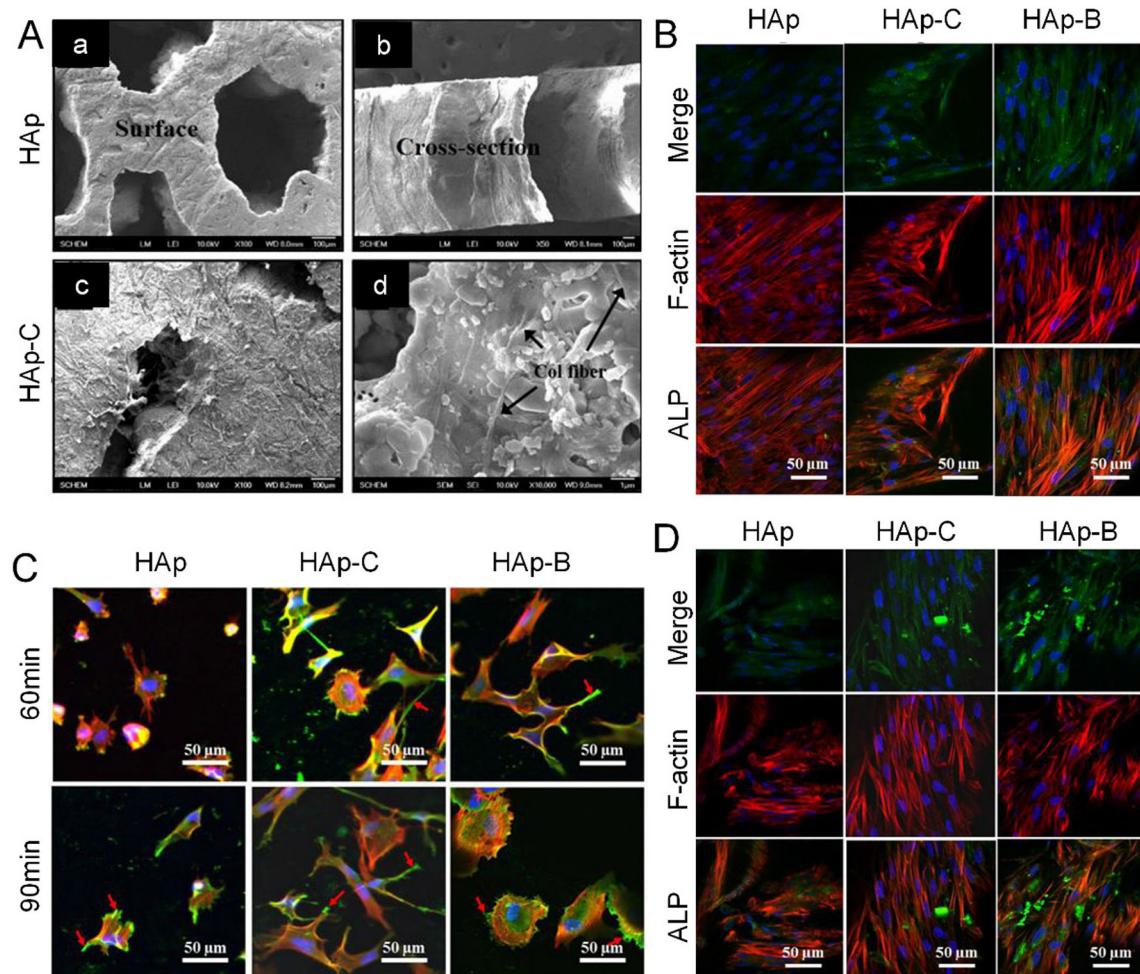


Fig. 9. (A) Electron microscopy images of HAp and HAp-C. (B) Alizarin red staining and ALP immunofluorescence detection of human adipose-derived stem cells (hADSCs) after 14 days of incubation. (C) Vinculin distribution of hADSC on HAp, HAp-C, and HAp-B after an incubation period of 60 and 90 min using confocal microscopy. (D) Alizarin red staining and ALP immunofluorescence detection of hADSCs after 21 days of incubation. HAp: Hydroxyapatite, HAp-B: BMP-2-conjugated, HAp-C: collagen-coated. Adapted from a previous study, with permission [198].

challenge because of the extremely long and complex healing process of contaminated and infectious bone defects [204,205]. Silver (Ag) is resistant to a broader range of bacteria because it can bind and destroy various components related to bacterial structure and metabolism [206]. However, Ag lacks osteoinductive properties, so it needs to be combined with osteogenesis-inducing substances. Sun et al. prepared collagen scaffolds composed of Ag and BMP-2 by freeze-drying methods [207]. When the concentration increased to 10 µg/ml, the proliferation of *Staphylococcus aureus* in the composite scaffolds was inhibited entirely, which indicated that the original antibacterial activity of *Staphylococcus aureus* was maintained after developing the Ag-coated collagen scaffolds. During the 7-day culture period, the BMSC proliferation rate on the scaffolds was only slightly different among the three groups, indicating that Ag had low cytotoxicity to cells other than bacteria.

Alendronate is a type of bisphosphonate containing a primary amino group, which is the first-line drug for the clinical treatment of osteoporosis. Studies have shown that alendronate promotes osteogenic differentiation of MSCs and inhibits osteoclast formation [208]. Functionally, the drug can rebalance the bone metabolism of osteoporosis. Zeng et al. encapsulated alendronate in a collagen/graphene oxide complex to accelerate bone regeneration [209]. CT scans showed that the volume of new bone in the defect site with 0.05% graphene oxide was almost three times that of other groups. Furthermore, CT and histological examination of

the femur demonstrated that this complex reduced the number of osteoclasts and inhibited the systemic bone loss in rats with osteoporosis. The drug release rate of collagen-based scaffolds may be related to its porosity and degradation rate. High porosity increases the surface area of drug dissolution, thus enhancing drug release. Drug loading by electrostatic adsorption is a common method, so the water environment around the scaffold may destroy the gravity and promote drug release.

5.2.3. Collagen-based scaffolds loaded with cells for osteogenesis and vascularization

Dental pulp stem cells (DPSCs) and BMSCs have similar immunomodulatory properties in the allogeneic and xenogeneic applications. It has been proved that DPSCs transplanted into rat skull defects can differentiate into osteoblasts without any graft rejection [210,211]. Chamieh et al. transferred DPSCs into collagen scaffolds to repair the rat skull defect model [212]. The type I collagen hydrogel solution was mixed with rat DPSCs (rDPSCs) at low temperatures. After polymerization, it was placed on blotting paper, nylon and stainless-steel mesh, and dense collagen scaffolds with fibrous collagen density greater than 10%. Compared with the acellular scaffolds after the operation, the scaffolds implanted with rDPSCs showed a higher amount of calcification in the reconstructed defects (Fig. 10). The parietal bone defect implanted with rDPSCs or acellular densified gel scaffolds showed gradual new

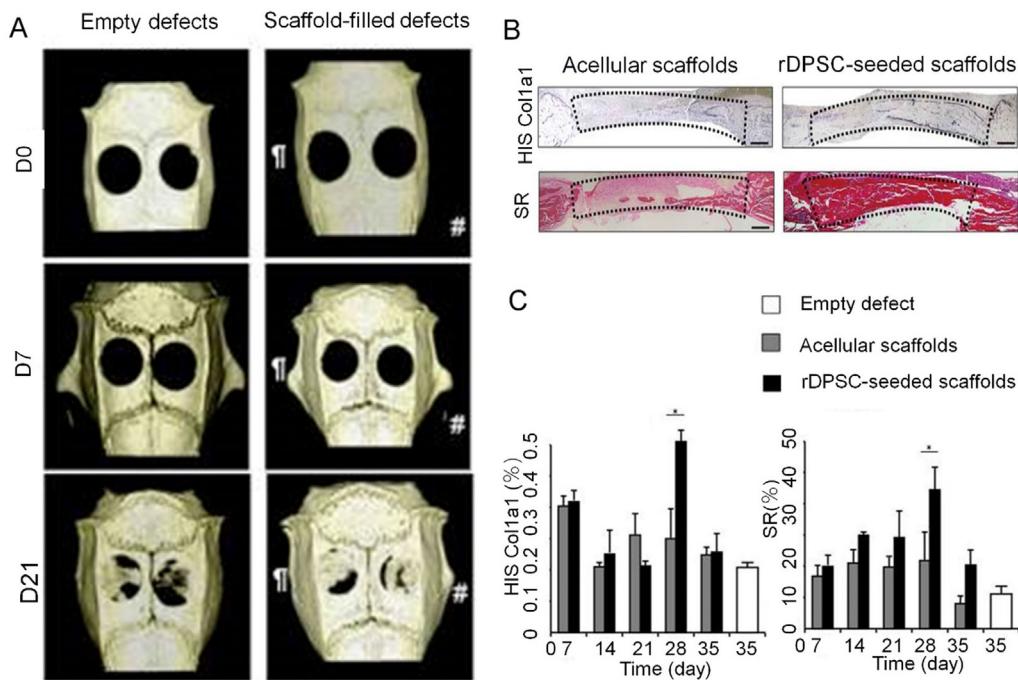


Fig. 10. (A) A micro-CT analysis of a rat skull (3D rendering) at days 0, 7, and 21. (B) Representative images at day 28 (post-operation) of the collagen component. (C) Quantification from days 14 to 35 of HIS Col1a1 and Sirius red (SR) staining. Adapted from a previous study, with permission [212].

bone formation from the edge and center of the defect. Strong collagen expression was observed in the scaffolds implanted with rDPSCs, especially in the inner and outer periosteal sides of the newly-formed bipolar cranium, while only a weak Col1a1-related signal was observed in the acellular scaffolds. The regulation of stem cell differentiation and function is an essential step in developing biological activity and clinical application of bone regeneration and function. Allografts have been used for bone repair and regeneration. Collagen and HA complexes promote the proliferation and differentiation of BMSCs and play an important role in the physiological process of osteogenesis.

In another study, BMSCs were implanted into collagen-hydroxyapatite-PCL (HAP-col-PCL) composite scaffolds to study the effect of BMSCs on local bone defect repair in rats [213]. The results showed that compared with COL-PCL or HAP-PCL scaffolds, 3D HAP-COL-PCL scaffolds with BMSCs had a greater effect of promoting new bone formation. Quantitative micro-CT analysis showed that the bone mineral density and bone volume (BV)/tissue volume (TV) ratio of the HAP-COL-PCL scaffolds group were significantly higher than those of the other two groups. Similarly, the combination of these materials can also treat avascular necrosis of the femoral head, a common and irreversible orthopedic disease resulting from trauma and improper use of hormones [214]. Conventional treatments such as core decompression can only delay the progress of the disease; therefore, the current problem is how to perform early treatment and avoid joint replacement. A new type of cellular scaffold comprised of nano-hydroxyapatite, collagen, and polylactic acid (nHAC/PLA) is implanted into the bone tunnel of the core decompression. The repair rate of the defects with implanted cells was about 10% higher than that without implanted cells, and the amount of vascular regeneration was also significantly higher than that of other groups.

It has been proved that stem cells are more sensitive to mechanical stimulation than somatic cells, and biomechanical signals are crucial for regulating the phenotypic differentiation of stem cells [215]. However, traditional tissue engineering strategies often lack effective mechanical stimulation in cell culture. Many

studies have shown that stem cells encapsulated in scaffolds stimulated by dynamic loading are more similar to natural tissues [216]. The natural frequency and strength of the 3D-cultured BMSC collagen scaffolds were simulated by dynamic mechanical loading [217]. Compared with the static culture group, dynamic mechanical loading promoted BMSC adhesion, uniform proliferation, and chondrogenic differentiation. Even with slight contraction, there was a significant improvement in the mechanical strength of the BMSC collagen scaffold, which better simulated the structure and function of natural cartilage.

A summary of the abovementioned studies in this section are presented in Table 5.

6. Perspectives

Collagen is an essential component of the natural bone matrix, and it is used for bone regeneration and biomimetic applications. Compared with the low bioactivity of most biomimetic materials, collagen has good biodegradability and biocompatibility, and sufficient plasticity. Therefore, the applications of collagen vary widely.

Collagen is rich in sources and can be extracted from different species (such as mammals, marine organisms and invertebrates). Natural collagen has weak immunogenicity and can be further reduced by chemical modification. Collagen regulates the activity of osteoblasts and osteoclasts through a variety of signaling pathways, and promotes the repair of bone defects. The development of collagen extraction technology has also created conditions for the wide application of collagen. According to the current research, collagen based biomimetic materials can be modified by a variety of materials to improve their biological properties. The application of collagen can be divided into two commonly used forms: flexible hydrogel and rigid scaffold. Chitosan, hyaluronic acid and alginate have biocompatibility, hydrophilicity and biodegradability. Collagen based biomimetic materials can be prepared by combining chitosan, hyaluronic acid and alginate with collagen in different proportions. In addition, HA and bioactive glass are expected to

Table 5

Summary of collagen-based hydrogel synthesized with cells.

Differentiation Direction	Cell Type	Materials	Identify Methods			Reference
			Staining	RT-PCR	Others	
Osteogenesis	rDPSCs	Collagen	Toluidine Blue staining, Von Kossa staining, Sirius red staining, Immunohistochemistry		Flow cytometry, Micro-CT	[212]
Osteogenesis	BMSCs	Collagen, HA, PCL	Sequential fluorescent labeling, Van Gieson's picrofuchsin staining, Immunohistochemistry		Micro-CT	[213]
Chondrogenesis	BMSCs	Collagen	Live/dead staining, Toluidine blue staining, HE staining	COL1A2, COL2A1, COL10A1, AGG, SOX9	SEM, Mechanical analysis	[217]

improve the mechanical properties and structural stability of the materials. Hydrogels are usually mixed with aqueous solutions and cross-linking agents of different materials, while rigid scaffolds are synthesized by cyclic freeze-drying and biomimetic mineralization. By adjusting the kinds and proportions of different materials, collagen-based hydrogel, which is suitable for osseointegration viscosity and rheology, were obtained, and the porous structure of collagen-based hydrogel enables them to exchange substances with blood, so that cells can obtain continuous nutritional supply. In addition, combined with other materials, collagen-based scaffolds have stronger compressive strength, stiffness and pore structure, which can significantly improve the bone repair effect. Bioactive substances, including chemicals, cells and growth factors, can promote the osteogenesis and angiogenesis of scaffolds through a variety of signaling pathways. For example, BMP-2 can promote BMSCs to differentiate into osteoblasts by activating MAPK and SMAD pathways. Collagen-based hydrogel is usually used as a delivery platform. With the degradation and diffusion of the gel, bioactive substances can be released to the local part continuously. By changing the proportion of different materials, the degradation rate of collagen-based hydrogel can be changed and the release rate can be controlled. Through physical mixing and electrostatic adsorption, bioactive materials were loaded into collagen-based scaffolds to enhance local bone regeneration.

So far, a variety of collagen complexes for bone regeneration have been studied in vitro. However, there is still a lack of complete in vivo experiments to verify the practicability of these scaffolds. In addition, it is still a challenge to manufacture composites that can meet all the desired properties including porosity, pore size, biocompatibility, mechanical integrity, structural stability, bone conductivity and osteoinductivity to achieve perfect bone regeneration. At present, there is no method that can perfectly simulate the natural state of bone regeneration and the internal and external multi-layered complex structure of natural bone. With the development of bioprinting technology, tissue engineering and biomimetic mineralization, the clinical success of composite collagen-based materials in bone regeneration is just around the corner.

7. Conclusion

Collagen, as a natural biopolymer, plays an important role in bone regeneration. Collagen has the advantages of biocompatibility, biodegradability, non-toxicity and non-immunogenicity. However, its mechanical properties are very low. Because of its strong plasticity, collagen can combine with other materials to synthesize rigid scaffolds with high mechanical properties. In addition, collagen-based scaffolds can be surface modified by attaching bioactive substances to promote bone regeneration. Collagen based hydrogel is made up of collagen and other materials, which makes up for the problem of excessive collagen degradation and swelling. The degradation time of collagen-based hydrogel can be prolonged for several months according to different materials. Bioactive sub-

stances can be directly loaded into hydrogels and released at appropriate concentrations to promote bone regeneration locally. Overall, our study concludes that collagen is a promising biomimetic material for bone tissue engineering.

CRediT authorship contribution statement

Youbin Li: Conceptualization, Investigation, Writing – original draft. **Yuzhe Liu:** Data curation, Writing – review & editing. **Ronghang Li:** Validation, Methodology. **Haotian Bai:** Writing – review & editing, Methodology, Conceptualization. **Zhengqing Zhu:** Conceptualization, Methodology, Supervision. **Liwei Zhu:** Visualization, Software. **Chenyi Zhu:** Conceptualization, Supervision. **Zhenjia Che:** Data curation, Validation. **He Liu:** Conceptualization, Validation, Methodology. **Jincheng Wang:** Visualization, Software. **Lanfeng Huang:** Conceptualization, Writing – review & editing.

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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