Materials Today Bio 20 (2023) 100660

Contents lists available at ScienceDirect

Materials Today Bio

journal homepage: www.journals.elsevier.com/materials-today-bio

Functionalization of biomimetic mineralized collagen for bone tissue engineering

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ARTICLE INFO

Keywords:
Mineralized collagen
Biomimetic
Functionalization
Bioactive factors
Osteogenesis
Bone tissue repair

ABSTRACT

Mineralized collagen (MC) is the basic unit of bone structure and function and is the main component of the extracellular matrix (ECM) in bone tissue. In the biomimetic method, MC with different nanostructures of neo-bone have been constructed. Among these, extra-fibrous MC has been approved by regulatory agencies and applied in clinical practice to play an active role in bone defect repair. However, in the complex microenvironment of bone defects, such as in blood supply disorders and infections, MC is unable to effectively perform its pro-osteogenic activities and needs to be functionalized to include osteogenesis and the enhancement of angiogenesis, anti-infection, and immunomodulation. This article aimed to discuss the preparation and biological performance of MC with different nanostructures in detail, and summarize its functionalization strategy. Then we describe the recent advances in the osteo-inductive properties and multifunctional coordination of MC. Finally, the latest research progress of functionalized biomimetic MC, along with the development challenges and future trends, are discussed. This paper provides a theoretical basis and advanced design philosophy for bone tissue engineering in different bone microenvironments.

1. Introduction

Natural bone is a mineralized hard tissue, consisting of an extracellular matrix (ECM) and bone progenitor cells, osteoblasts, osteoclasts, and bone cells. Embedded therein [1] (Fig. 1). The ECM is composed of organic-inorganic composite materials with mineralized collagen (MC) fibers and a complex hierarchical structure. Calcium phosphate, mainly composed of hydroxyapatite (HA), is the principal inorganic component of vertebrate bones, that constitutes nearly the 65% of bone weight [2]. HA has a hexagonal crystal system, which has a flexible and stable structure and composition, allowing the substitution of a variety of metal ions [3–5]. Impure ions, such as carbonate, sodium, and magnesium, can replace phosphate and hydroxyl sites [6–8], resulting in poor crystallization, calcium deficiency, and carbonization of the HA [9]. Organic components account for approximately 30% of bone weight [10], among which type I collagen is the most abundant in bone tissue. The triple helix structure of collagen I is usually heterotrimeric and composed of two identical a1(I) chains and one a2(I) chain. Individual triple helix molecules of collagen undergo self-assembly to form fibrils, and the ordered arrangement forms an observable periodicity known as the D-band, measuring 67 nm with a gap between two consecutive collagen molecules measuring 36 nm, hence the name, “gap region” [11]. Additionally, collagen fibers provide more nucleation sites for apatite crystals to aggregate [12,13], which guides the growth of mineral crystals and aligns them along the long axis of the fiber, resulting in a larger particle size [14]. MC is assembled by the orderly deposition of nano-hydroxyapatite (nHAP) across the collagen Iorganic matrix, and is the most prominent level in the complex hierarchical structure of natural bone. MC provides the nanostructure base for the excellent mechanical and biological properties of bone [15]. According to how the HA is distributed relative to the collagen fibrils, MC can be divided into intra-fibrous MC (IMC) and extra-fibrous MC (EMC) [16]. IMC minerals are deposited within the collagen matrix, while EMC minerals are randomly deposited on the surface.

Throughout the human life cycle, bone tissue continues to remodel to adapt to mechanical stress and maintain skeletal tissue integrity. Minor bone defects can heal by themselves through bone reconstruction, while large traumatic injuries and defects caused by tumors, congenital

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https://doi.org/10.1016/j.mtbio.2023.100660
Received 28 February 2023; Received in revised form 18 April 2023; Accepted 4 May 2023
Available online 6 May 2023

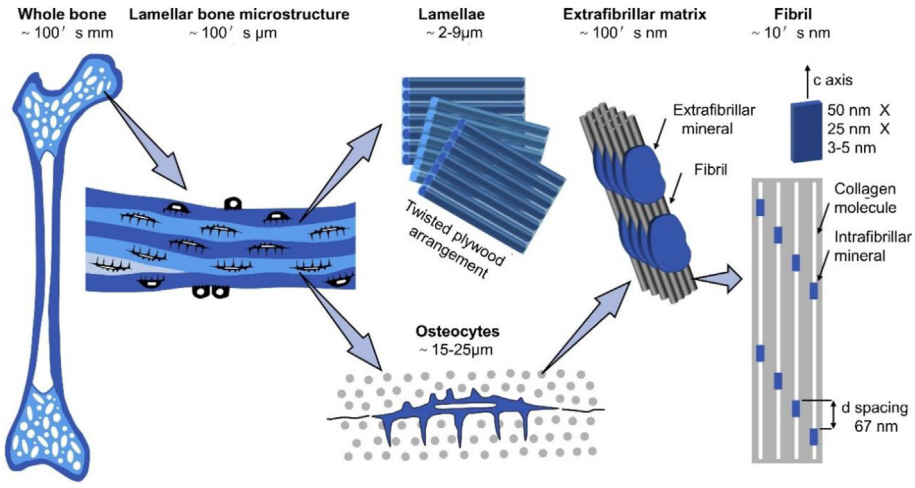


Fig. 1. The multiscale structure of bone [1]. Reproduced with permission [1]. Copyright 2019, Elsevier.

diseases, and infectious diseases usually require the intervention of surgery and bone substitutes [17]. Autologous and allogeneic bone are common alternative materials to aid the healing of bone defects. However, the sources of autologous bone are limited and are prone to infection-related complications at the sampling site following transplantation. In addition, the use of bone allografts is not widespread in clinical practice due to the risks of infection and immune rejection [18,19]. Bone tissue engineering is an innovative approach to repairing and regenerating bone tissue, and a significant advancement in this field is the use of autologous bone replacement. Thus, it is imperative to identify suitable alternatives or substitutes to bone transplants.

The hierarchical structure of bone, consisting of nine levels, provides the skeletal system with the ability to bear weight and withstand mechanical stresses [20]. Suitable alternatives or substitutes for bone transplants should have similar mechanical strength and degradation rates to natural bone, while also mimicking the composition and structure of the extracellular matrix (ECM), and creating a microenvironment that is conducive to the growth of cells and tissues [21–26]. Biomimetic materials, such as MC, are engineered to mimic the first two levels of this hierarchy - the chemical composition and structural elements [27]. They possess excellent biocompatibility, biodegradability, low antigenicity, and compositional and structural flexibility, which promote adhesion, proliferation, and differentiation of pre-osteoblasts or stem cells *in vitro*, as well as cell migration ratios [21,28–31]; enhances osteogenesis and angiogenesis *in vivo*; and promotes the repair of bone defects and the osseointegration of implants [31–36]. Compared with pure collagen, HA, and tricalcium phosphate (TCP) bone scaffold materials, MC can better promote osteogenic differentiation, induce ECM secretion and mineralization, stimulate angiogenesis, and ultimately promote osteogenesis [24,28,37–41]. At present, there are numerous MC products composed of collagen/hydroxyapatite (COL/HA), some of which have been commercialized and approved by regulatory agencies for clinical application [42]. In terms of repairing minor bone defects and bone regeneration, MC has virtually achieved the same effect as autologous bone [43,44]. However, for the repair of larger and more complex bone defects, MC materials with better biomimetic properties, and stronger functionality is often required, which is a hotspot of research for MC bone replacement materials. In principle, if only the structure and morphology of MC are changed, the biological-enhancing effects of these materials are limited. To enhance the function of MC, it is often necessary to introduce additional therapeutic stimuli (e.g. cells, growth factors, bioactive elements, drugs.), rapidly promote the deposition of new bone, and ensure the formation of an adequate vascular network to achieve rapid regeneration of endogenous tissue at the injured site [22,45–48].

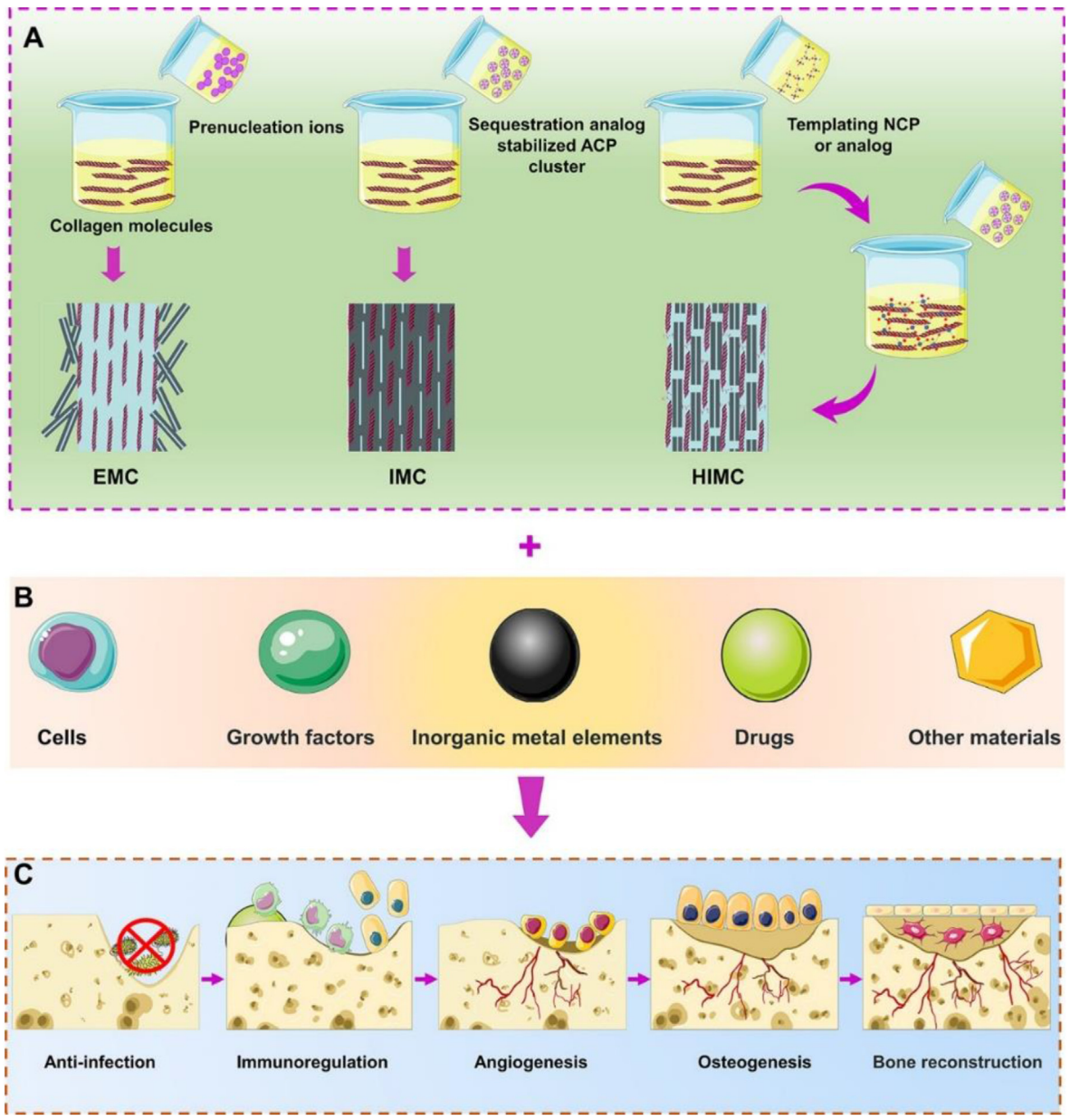
Currently, research on MC has progressed from pure and composite biological materials to multifunctional coordination materials that can incorporate specific functional cells, bioactive factors, and drugs for enhanced therapeutic efficacy (Scheme 1). Existing reviews on MC mainly focus on the mechanism of biomineralization, classical and non-classical crystallization theories, *in vitro* mineralization of collagen, and preparation of the MC scaffolds and MC [15,16,49–51]. The construction of more coordinated and multifunctional MC composites for the repair of complex bone defects are a current research hotspot, with a growing number of related studies. We present the preparation strategies and biological performance of MC. We then summarize the multi-functionalized programs of MC, and subsequently focus on two major issues: improving the osteogenic property and versatile coordination potential of MC. Finally, we review related studies, discuss the current problems of MC materials, and provide an outlook for future development trends of more coordinated and multifunctional MC composites. This will provide an advanced treatment strategy and theoretical basis for the application of MC in large or complex bone defects.

2. Biomimetic collagen mineralization

The hierarchical structure and chemical composition of native bone tissue have been described earlier to further our knowledge and understanding of MC. Here, we will focus on advanced strategies for preparing MC with different nanostructures (as shown in Fig. 2) and explore their biological functions.

2.1. Fabrication of MC with different nanostructures

Type I collagen, minerals, and non-collagen analogs (NCP) are the three basic elements for the preparation of MC [15]. Based on the differences in minerals, the preparation of MC can be divided into two methods: direct mineral addition and in situ mineralization [49]. The former is described as directly adding the minerals to the collagen solution. Then, the MC scaffold is prepared by electrospinning, freeze-drying, or coating methods. Finally, MC, which mimics natural bone matrix in composition and structure, is obtained. This is the simplest method for the preparation of MC. During in situ mineralization, the calcium and phosphate ions are introduced into the collagen solution instead of mineral crystals, or a pre-formed collagen scaffold is immersed in a solution containing both mineral ions. *In situ* deposition is a research hotspot in the preparation of MC, and MC with nanostructures similar to natural bone has been successfully prepared [52–55]. *In situ* deposition contains a variety of MC preparation strategies, and different strategies provide diverse forms of I-col binding with minerals, which have been comprehensively described by Li et al. [49]. Here, we introduce the



Scheme 1. Schematic illustration of functional MC synthesis strategies, modification, and some of the active factors and cellular aspects that take part in the bone repair processes in each stage.

preparation methods of EMC, IMC, and HIMC, which has been well investigated. This will further facilitate our understanding of the nanostructure and function of MC.

Classical ion-mediated crystallization strategy (CIMC) is the most common method to prepare EMC [56–59]. The collagen solution is mixed with a mineralization solution containing calcium and phosphate ions, the pH is then adjusted to 7.4 to produce a neutral solution, and the EMC is obtained by incubation at 37 °C for a period of time without external force. In this process, the self-assembly process of collagen fibers and mineralization occurs simultaneously. However, the amorphous calcium phosphate formed during the process is often too large to penetrate into the collagen fibers. As a result, larger calcium phosphate particles only attach to the surface of the collagen. This process is simple and easy to manipulate, but it is difficult to replicate the nanostructures of natural bone.

With the growing interest in biomineralization processes, polymer-induced liquid-precursor pathway (PILP) [60] strategies have received much attention. On the basis of CIMC [61], acidic polymers (i.e., NCP analogs, also known as isolation analogs, such as polyacrylic acid [PAA] and polyaspartic acid [PAsP]) are introduced into a supersaturated mineralization solution to bind and isolate calcium ions, delay crystal nucleation and growth, form stable and highly hydrated amorphous precursors, and prevent amorphous calcium phosphate precursors from aggregating and automatically transforming into apatite before entering the collagen fiber gap [62,63]. Thus, a continuous apatite band is formed inside the collagen fiber, and IMC is obtained [55,64–66].

Inspired by the dual function of matrix phosphoproteins in the process of biological mineralization [67], some researchers have introduced polyphosphates—such as polyethylene phosphonic acid, sodium trimetaphate (STMP), and sodium tripolyphosphate (TPP)—into the mineralization system on the basis of the PILP strategy as another NCP analog (also known as template analog). This is considered to be the dual biomimetic analog strategy (DBA), whereby polyphosphate is capable of efficiently binding with collagen fibers via electrostatic interactions,

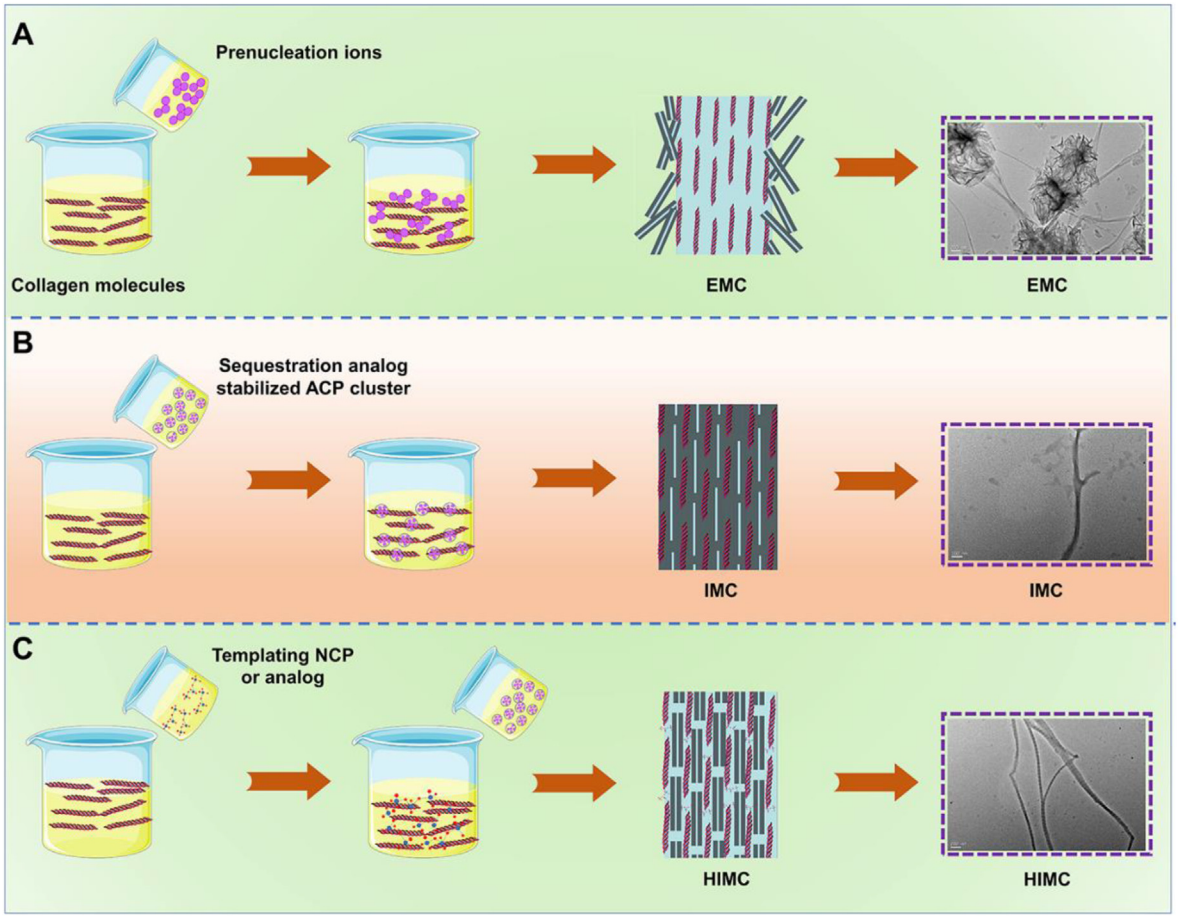


Fig. 2. Common methods used to prepare MC with different nanostructures and the corresponding transmission electron microscopy (TEM) images. (A) The preparation process of EMC by classical ion-mediated crystallization strategy. (B) The preparation of IMC by procollagen 1 intact *N*-terminal (PINP) pathway. (C) The preparation process of hierarchical, intrafibrillarly MC (HIMC) by dual biomimetic analog-based bottom-up strategy.

inhibit the continuous growth of apatite in the overlapping area, and form an in-fiber MC with hierarchical apatite structure of natural bone tissue. Some authors refer to this as HIMC. TEM analysis of HIMC reveals parallel ribbon-like particles aligned with the long axes of the collagen fibrils [23,52,68–71]. The regulation of biomimetic mineralization processes by NCP analogs is a key step to reproducing the nanostructures of natural bone. Recent studies have demonstrated that periodic fluid shear stress (FSS) can replace polyacrylic acid (PAA) and induce highly-arranged IMC and HIMC in the presence of TPP [72,73]. Moreover, periodic FSS has been shown to improve the hydrophilicity, enzymatic stability, and crystal conversion of mineralized collagen [74]. In addition, other substances such as polyamide dendritics [75,76], sodium alginate [77], chitosan [21,53], sodium citrate [78], succinic acid [79], alkaline phosphatase (ALP) [80], and osteopontin (OPN) [81] have also been found to regulate the process of biomimetic mineralization *in vitro*. Moreover, some specially modified mesoporous silica nanoparticles and hollow mesoporous zirconia nanocapsules can realize the loading and delivery of ACP [54,82] in the preparation of IMC. In general, the direct mineral addition method and the CIMC strategy are simple and time-savings, and are commonly used in bone tissue engineering studies. Biomineralized hard tissues, such as bones and teeth, possess exceptional mechanical properties due to their unique architecture and hierarchically arranged nanostructures [83,84]. MC fabricated by the PILP or DBA strategies have a similar nanostructure to natural bone tissue and exhibit better mechanical properties and bioactivity compared to traditional MC. However, the preparation process is complicated and time-consuming.

2.2. Bioactivity of MC with different nanostructures

Variations in preparation methods, material sources, and processing parameters can lead to changes in the nanostructure of MC [74]. These changes, in turn, affect the mechanical properties, degradability, and bone-inducing ability of MC both *in vitro* and *in vivo*. Factors such as cell proliferation, osteogenic differentiation, focal adhesion, macrophage polarization, host MSC recruitment, new bone generation, and capillary formation can be impacted. Different types of nanostructures can have varying effects on these factors, with IMC and HIMC demonstrating greater similarity to natural bone than other nanostructures (Table 1) [23,52,85]. The nanostructures and degradation properties are similar between HIMC and natural bone, and HIMC is capable of regulating stem cell recruitment and promoting osteogenic differentiation by providing an optimized microenvironment, which facilitates the growth of new bone.

HIMC has better biocompatibility and osteogenic activity *in vivo* and *in vitro* than EMC and IMC [90] (Fig. 3). HIMC and IMC have significantly increased Young's elasticity compared with EMC, which significantly promotes the adhesion, proliferation, differentiation, and cytoskeletal arrangement of MG63 cells, MC3T3-E1 osteoblasts, and mesenchymal stem cells, and promotes the expression of osteogenic-related genes. Finally, HIMC and IMC can significantly promote new bone formation and bone defects repair [21,31,86,90,91].

IMC can also promote the expression of the osteoprotegerin (OPG) gene in HMSCs, inhibit the generation of osteoclasts, and then affect bone

Table 1
Properties of MC with different nanostructures.

Experimental group	Control group	Cells or animal model	Major findings	Reference
HIMC	EMC; collagen	MG 63	HIMC possesses better mechanical and biological properties, specifically cell proliferation, differentiation, focal adhesion, and cytoskeletal arrangement.	[86]
HIMC	Collagen; EMC	MC3T3-E1	Pure collagen scaffolds had the highest rate of proliferation.	[71]
EMC	HA	hMSCs	Promoted cell proliferation and osteogenic differentiation of hMSCs.	[40]
IMC	Collagen	hMSCs	Both support proliferation, osteogenic differentiation, and mineralization of hMSCs, with IMC having a more pronounced positive effect.	[87]
IMC	β-TCP, collagen	Critical-sized rodent mandibular defect model	Activate more bone-forming cells and stimulates more vascular tissue ingrowth. Induces ECM secretion and mineralization of rBMSCs.	[24]
IMC	EMC	THP-1, critical-sized rodent mandibular defect model	Promotes more new bone formation and had more M2- like macrophages. Highly express IL-10 and arginase-1.	[88]
HIMC	EMC	Critical-sized rodent mandibular defect model, THP-1, hBMSCs	Recruits host MSCs and promotes endogenous bone regeneration by immunomodulation of macrophage polarization through IL-4.	[31]
IMC	EMC, HA	Critical-sized bone defect in the rat femur	Enhances bone regeneration via activation of the Wnt signaling pathway.	[33]
EMC	Collagen	RAW264.7	Downregulates inflammation and innate immunity. Upregulates nucleosome assembly, megakaryocyte differentiation, and chromatin assembly.	[38]
IMC	EMC	BMSC	Promotes new bone generation and capillary formation.	[89]
IMC + Ti	EMC + Ti ,	Critical-sized bone defect in the rat femur	Promotes bone regeneration and osseointegration.	[32]

HCM, Hypoxic conditioned medium; EMC, Extra-fibrous mineralized collagen; IMC, Intra-fibrous mineralized collagen; HIMC, Hierarchical, intrafibrillarly mineralized collagen; hMSC, Human bone marrow mesenchymal stem cells; BMSC, bony marrow mesenchymal stem cells.

metabolism [37]. Filling MC around titanium implants or in 3D printed porous titanium alloy scaffolds can promote osseointegration [32,36]. Moreover, the application of IMC leads to better osseointegration compared with EMC. One study found that IMC significantly promotes bone regeneration by activating the Wnt signaling pathway (related factors Wnt5a, β-catenin, and Axin2), and guides the early process of bone regeneration by promoting the expression of genes related to inflammation, immune response, bone development, angiogenesis, neurogenesis, and Wnt signaling pathways [33]. Compared with HA, EMC can promote the proliferation and osteogenic differentiation of hMSCs through osteoblast differentiation and skeletal system development pathways, and facilitate the expression of osteogenic-related genes, such as BMP-2, COL1A1, and CTSK [40].

3. Recombination patterns of MC and active factors

The method of binding of the active factors and MC not only affects the sustained release effect of active factors, but also affects the biological activity. In turn, the addition of active factors may also affect the content and crystal morphology of HA in MC, as well as the nanostructure and degradation of MC. By reviewing past studies, we divided the binding modes of active factors and mineralized collagen into four categories: adsorption onto MC by immersion; binding to MC after surface modification; mixing with raw materials of MC; and polymer encapsulate (Fig. 4).

3.1. Adsorption onto MC by immersion

MC is a loose and porous nanobiomaterial and has a high surface area favorable for the adsorption of proteins or drug substances. *In vivo*, MC has the ability to naturally absorb and enrich BMP-2 and vascular endothelial growth factor (VEGF) [92,93]. Nano-HA is the main component of MC and is able to adsorb many proteins and other molecules, such as drugs and ions [94]. Ca^{2-} and $PO4^{3-}$ are protein-binding sites on the surface of CA-P, which provide the main driving force for protein adsorption [95,96], and interact with –OH, -NH2, and –COOH groups of growth factors by hydrogen bonds or electrostatic interaction [97,98]. The adsorption ability of drugs on MC may be related to the electrostatic energy of HA [99] and its chelation ability with calcium phosphate [100]. The *C*-plane surface of HA is negatively charged and the amino group of vancomycin is positively charged after protonation [101], so it can be adsorbed on HA [102]. Bisphosphonates (BPS) can specifically bind to HA substrates through stable bidentate structural connections formed by chelation between phosphonic acid groups and calcium ions [103,104]. R1 and R2 groups derived from quaternary carbon branches of BPS can also bind to HA through hydroxyl groups or hydrogen bonds, further increasing the binding affinity between them [105,106]. These distinct properties make MC an ideal drug delivery system for long-term controlled delivery of active factors (AFs) to promote bone tissue repair [107–110].

Impregnation adsorption is a common strategy for loading growth factors (such as rhBMP-2, VEGF, antibiotics, etc.) onto MC. Typically, it involves an initial burst release followed by gradual and sharp cumulative release. The burst release provides sufficient stimulation for cell proliferation and affects osteogenic differentiation in the early stage [111,112]. However, it should be noted that the higher initial burst release has the potential to shorten the release time and reduce the effect of the drug. This strategy has a simple process and minor effect on protein activity, which can minimize the complexity of structural design, reduce the manufacturing and regulatory burden related to development, and facilitate clinical translation [112].

3.2. Binding to MC after surface modification

Surface modification improves the surface activity of biomaterials and enables the AF to form a stable binding to biomaterials. Heparan sulfate proteoglycan is a key component of the bone ECM, and its functional component, glycosaminoglycan heparin, is a linear polysaccharide that can promote the proliferation and osteogenic differentiation of mesenchymal stromal cells cultured *in vitro* [113]. Heparin is capable of binding specifically to various AFs, including growth factors, cytokines, chemokines, and additional signaling molecules, and stabilizing them in materials to regulate their activity [114,115]. Biomaterial surfaces have been modified with heparin or heparan sulfate-mimetic molecules to control the release of the heparin-binding growth factor [113,116–120]. In the sustained release process *in vitro*, heparin-modified MC can achieve a higher sustained and controlled release of AFs compared with non-heparin-modified MC [102,119]. The rate of AF release can be regulated by changing the amount of heparin incorporation and the modification methods [119].

Compared with fresh VEGF, the biological activity of VEGF sustained release from heparin-free modified MC was decreased, while the

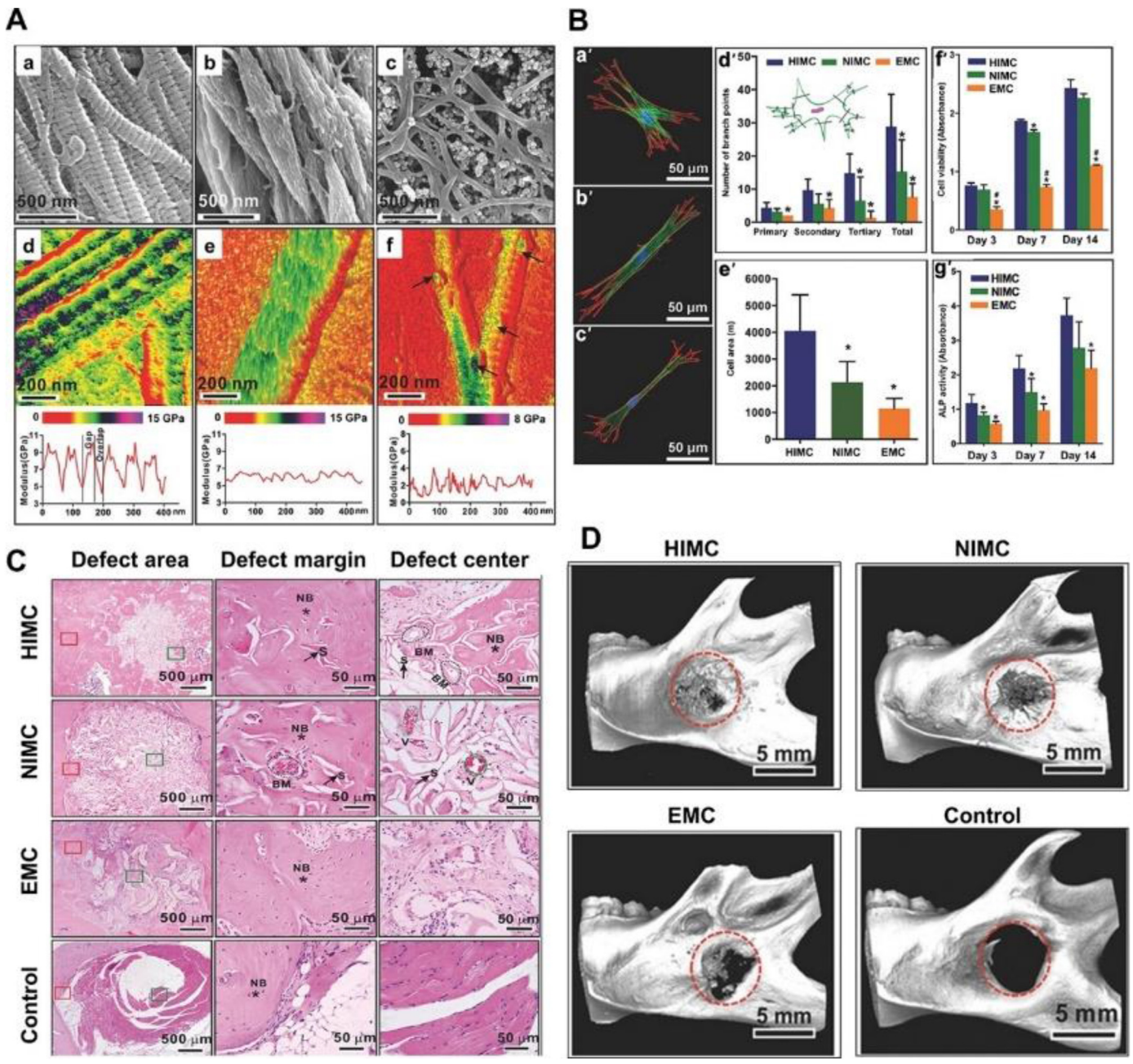


Fig. 3. Comparison of MC with different nanostructures. (A) Nanotopography (a–c) and nanomechanical (d–f) properties of MC with different nanostructures. Scanning electron microscope (SEM) image of HIMC (a), IMC (b) and EMC (c). Corresponding atomic force microscopy property maps and section analyses of Young's modulus of parts a–c, respectively. (B) rBMSC morphology (a′-c') after 1 d of culturing on the a) HIMC, b) IMC, and c) EMC. Cell morphology quantified for d′) the number of branch points and e′) the cell area in each group. f′) Cell viability and g′) quantitative results of ALP. (C) Representative HE staining images of mandibular defect areas in each group. (C) Micro-CT images of mandibular defect areas in each group. *$\alpha < 0.05$ versus HIMC; $^{\#}\alpha < 0.05$ versus IMC. Reproduced with permission [90]. Copyright 2016, John Wiley & Sons.

biological activity of VEGF sustained release from heparin-modified materials remained unchanged or was even enhanced [119]. When two or more bioactive factors of loaded heparin modified MC, the modification was generally accompanied by the initial sudden release of bioactive factors, followed by a slow release [118,121], and the sustained release process lasted for more than 6 weeks, which was considerably longer than in hydrogel [118,122]. Single and mixed factors show different release kinetics curves after loading, perhaps because different factors compete for free binding sites on the scaffold, and the binding efficiency of single factors is reduced by the elevated protein content in the mixture of bioactive factors. Therefore, the binding and release of AFs not only depends on the chemical interaction between them and the scaffold but also the interaction among the active factors [121].

In summary, functionalization of MC can be achieved through heparin modification and growth factor loading. The specific binding between heparin and AFs leads to a higher sustained release of the AF, while maintaining or enhancing its biological activity. Additionally, PDA's strong adhesion properties can be utilized to load AF onto biomaterials as an adhesive polymeric bridge. This approach has been used to create a bio-functionalized composite scaffold for osteonecrosis therapy that promotes osteo-conduction, angiogenesis, and a favorable metabolic microenvironment [123].

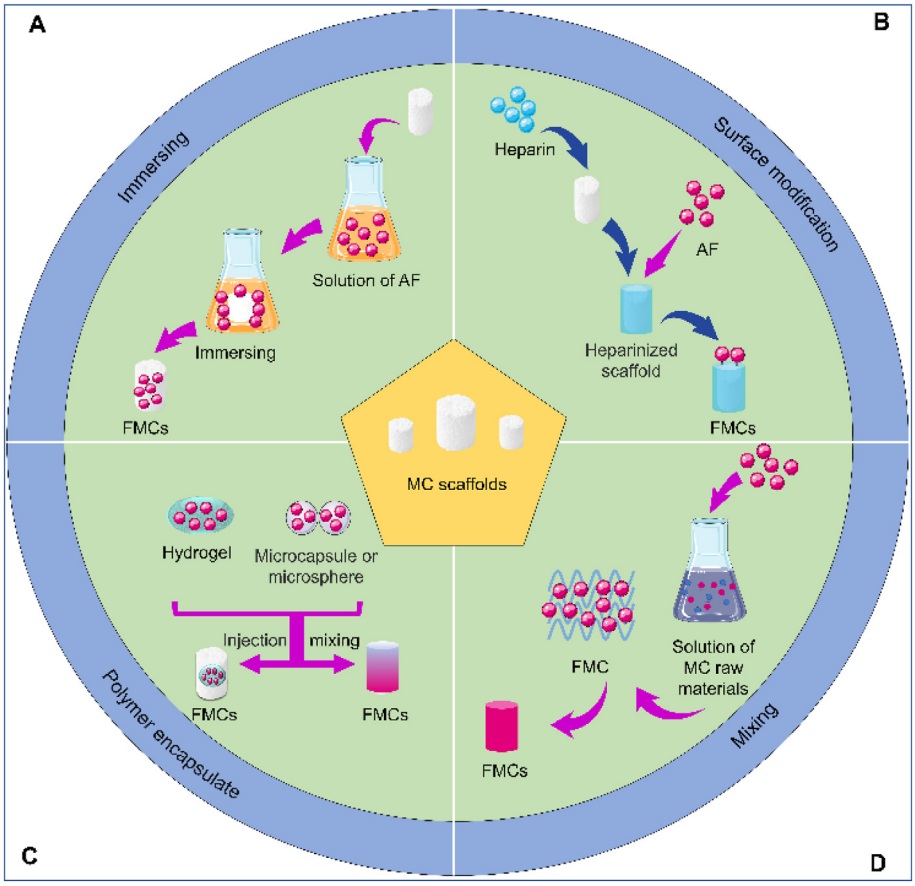


Fig. 4. Loading strategies of active factors on MC. (A) Highly efficient loading of active factor (AF) into MC by immersing in AF solution. (B) Heparin-modified surface of MC develops an affinity for AF. (C) AF was loaded onto MC scaffolds, and polymer was introduced into the scaffolds by injection or mixing. (D) AF was mixed with the raw materials of MC to form functionalized mineralized collagen (FMC). FMCS, functionalized MC scaffolds; FMC, functionalized MC; AF, active factors.

3.3. Polymer encapsulate

Prolonged-term retention and controlled release of AFs in bone defects is a critical prerequisite to ensure the osteogenic activity of bone repair materials [124]. There is a risk of uncontrolled release of growth factor (GF) and leakage to other areas of the body after the MC with adsorbed GF by immersion is implanted in the body [125]. The polymers have excellent bioactivity and biodegradability, and can be processed into injectable hydrogels [126,127], microcapsules [128], and microspheres [129–132] with certain mechanical strength. By surrounding the AF surface with biodegradable materials, we can regulate the release thereof [48,133], and the sequential release of AFs can be realized. Different polymers have been shown to have different delivery effects on AFs. For example, poly (lactide-*co*-glycolide) (PLGA) can release rhBMP-2 better than alginate [134], while alginate can realize the space-time controlled release of VEGF and maintain biological activity for a long time [135–137]. Furthermore, by incorporating magnesium particles into PLLA microspheres, the release of BMP-2 can be manipulated, achieving spatiotemporal co-release of magnesium and BMP-2 from the microspheres [132]. Functionalized mineralized collagen (FMC) can be obtained by injecting active factor (AF)-loaded hydrogel into MC composite or mixing AF-loaded microspheres with MC raw material. FMC composites can slowly release signaling factors in a humid environment *in vivo*, form a concentration gradient around the implant material, stimulate the directional migration of hBMSCs to the center of the scaffold, complete the recruitment of cells, and accelerate the healing of bone defects by promoting angiogenesis to provide oxygen and nutrition [127].

The sustained release process of AFs generally involves a combined diffusion/degradation mechanism [110,138]. Diffusion is regulated by the interaction of the materials with drug molecules, such as hydrophilic and hydrophobic interactions [139]. The gradual degradation of MC ensures the continuous and local release of AFs in the scaffold structure [140]. It should be noted that the invasion of bone related cells caused by some AFs accelerates the degradation of MC [111]. Alginate and hyaluronic acid hydrogels are able to delay the initial burst release of VEGF and prolong the release, and the introduction of heparin further enhances the retention of VEGF, with an approximately linear release of VEGF within 28 days [126]. In addition, both the pH of the microenvironment and the material concentration could alter the kinetics of AF release by affecting the interaction between the drug and the material or the solubility of the material [141].

3.4. Mixing with raw materials of MC

Mixing active ingredients (such as growth factors, inorganic metal ions, and drugs) and raw materials of MC to create functionalized scaffolds is another common loading strategy. RhBMP-2 has superior stability in an acidic environment [142,143], and can bind to HA through non-covalent bonds [97,99]. During preparation of MC, rhBMP-2 is added to a COL/HA slurry and the MC scaffold prepared by freeze-drying supports the controlled release of proteins, which attenuates the burst release, prolongs the release, and maintains the biological activity [48, 144]. In addition, the polyglutamic acid residue modification of GFs can provide an accumulated negative charge, which is conducive to electrostatic binding with positively charged HA, and significantly improves

the binding efficiency of AFs and MC [145,146]. Compared with the impregnation adsorption strategy, the incorporation of AFs in the MC preparation process makes the loading amount of drugs more controllable and can weaken the initial burst release, but various physical and chemical factors in the preparation process may affect the activity of the drugs [147].

HA in natural bone contains many doped cations or anions, which replace calcium or phosphate in the crystal lattice [148]. Compared with GFs, inorganic metal elements are inexpensive, relatively stable, and not easily inactivated. Some inorganic metal elements (such as strontium, zinc, iron, manganese, magnesium, and silver) are incorporated into MC materials to imitate the basic components of mammalian bones, which can improve the osteoinductivity of MC or sustain and anti-infective capacity [16,149–155]. The incorporation of inorganic metal elements has a significant effect on the degradation rate of materials, HA content, and microstructure and crystal morphology, but has an insignificant effect on the biomimetics mineralization process and the mechanical properties and pore structure of MC [152,153,155–158].

4. Enhancing osteogenic activity of MC

Osteogenic performance is an effective index to evaluate bone replacement materials in bone defect repair, reconstruction, and tissue regeneration. Here, we elaborate upon the aspects of MC loaded with cells, GFs, drugs, and inorganic metal elements. (Table 2).

4.1. loaded with osteoblast-related cells

As well as the ability to replicate, stem cells have multidirectional differentiation potential. Compared with stem cells, the proliferation and differentiation ability of osteoblasts is insufficient. In the process of bone repair, bone marrow mesenchymal stem cells can migrate to the bone defect and differentiate into osteoblasts, secreting bone matrix components and playing an important role in bone regeneration [161]. Stem cell-based tissue engineering has great potential to regenerate damaged tissue. MC has superior biocompatibility and can be used as a cell carrier; The combination of both work together to significantly promote bone regeneration *in vivo* [23]. In a study of skull bone defect repair models in miniature pigs, compared with the cell-loaded HA scaffold, autologous periodontal ligament stem cell (PDLSC)-loaded IMC showed better bone regeneration and the deposition of large amounts of new bone with nanostructures. Furthermore, nanomechanical properties and blood vessels similar to natural bone were observed, and the expressions of Runt-related transcription factor 2 (Runx2) and transcription factor Osterix were high [22]. Autologous adipose-derived mesenchymal stem cell (ADMSCs)-loaded nano-hydroxyapatite-collagen-polylactic acid (NHAC-PLA) achieved similar fusion effect as autologous iliac bone transplantation (ACB) in the process of spinal fusion in rabbits [162]. Despite the promising potential of stem cells, there is currently no consensus on their optimal conditions for use. Therefore, the establishment of uniform standards and requirements for the use of stem cells is crucial for their development and application.

4.2. Addition of GFs

MC is an effective carrier of various GFs, which release a variety of signal factors *in vivo* to attract and stimulate surrounding host tissue cells and promote the inward growth of osteoblasts and the formation of the vascular network in the scaffold, ultimately promoting osteogenesis [108,110,112,144,145,163]. Of the bone GFs discovered, BMP-2 is the strongest growth factor in promoting bone formation. RhBMP −2 has been approved by the Food and Drug Administration and applied to clinical treatment of various orthopedic and stomatological diseases [164]. However, there is growing evidence suggesting that the excessive dosage application of rhBMP-2 and the uncontrolled and non-targeted delivery after implantation lead to a series of complications [164–168]. Some researchers have incorporated rhBMP-2 30 times lower than the clinical gold standard, INFUSE®, during MC preparation. In trials, this was not accompanied by an obvious initial burst release *in vitro*, and only about 25% rhBMP-2 was released in 21 days, which significantly improved the degree of skull defect healing without bone abnormalities or the resorption of the adjacent bones [144]. Low concentrations of rhBMP-2 have also shown strong osteogenic ability in large animal models [160] (Fig. 5). MC scaffolds were loaded with PLGA-encapsulated rhBMP-2 particles, which were shown to continuously release bioactive rhBMP-2 and exhibit exceptional bone regeneration and healing properties *in vivo* [134].

The functionalization effect of single growth factor is lower than that of combinations of different growth factors [169,170]. Heparin-modified MC scaffolds were loaded with different doses of BMP-2 and chemokine stromal cell-derived factor-1α (SDF-1α). *In vivo*, SDF-1a enhanced the osteoinductive potential of the low concentrations of BMP-2, producing a regenerative potential similar to that of high-dose BMP-2, thus avoiding the adverse effects produced by excessive doses of BMP-2 [118]. BMP-2-derived peptides are peptides that retain BMP sequence inducing osteoblast differentiation. Various BMP-2-derived peptides have been designed and show significant osteoinductive ability [112,171]. *In vivo*, MC scaffolds loaded with BMP-2 derived peptides can control the

Table 2
Enhancement of the osteogenic activity of MC.

Classification of MC	Synthetic strategy	Active factors	Loading strategy	Cells or animal model	Major findings	Reference
HIMC	DBA	Iron, manganese	Mixing	Calvarial defect model in rats	Promotes osteogenic differentiation of BMSCs and bone regeneration loaded with fresh bone marrow cells.	[152]
EMC	CIMC	GAG, BMP-2	Mixing	BMSC, Parietal defect in rabbits	Induces healing of cranial defects without addition of expanded stem cells or exogenous growth factors.	[47]
EMC	CIMC	GAG	Mixing	hMSCs	Activates the Wnt and mechanotransduction pathways and promotes osteogenesis.	[159]
EMC	CIMC	GAG, zinc	Mixing	pASCs	Promotes successful growth and pro-osteogenic capacity of pASCs.	[155]
MC	DMA	β-tricalcium phosphate, BMP-2	Mixing; immersing	Male beagle dogs, Saddle-type alveolar defects	HAp/TCP/Col with 0.2 mg/ml rhBMP-2 manifested more and faster new bone formation with better implant stability.	[160]
MC	DMA	rhBMP-2	Polymer encapsulate	MC3T3-E1, critical-sized calvarial defects in rats	Enhanced pro-osteogenic effect *in vitro* and *in vivo*.	[134]
HIMC	DBA	hUCMSC		hUCMSC; femoral condyle defect models in rabbits	Promotes healing speed of bone defects *in vivo*.	[23]

DMA, direct mineral addition method; CIMC, Classical Ion-Mediated Crystallization Strategy; DBA, Dual Biomimetic Analog Strategy; PILP, Polymer-Induced Liquid-Precursor Pathway; hUCMSC, human umbilical cord mesenchymal stem cell; GAG, Glycosaminoglycan; pASCs, porcine adipose-derived stem cells.

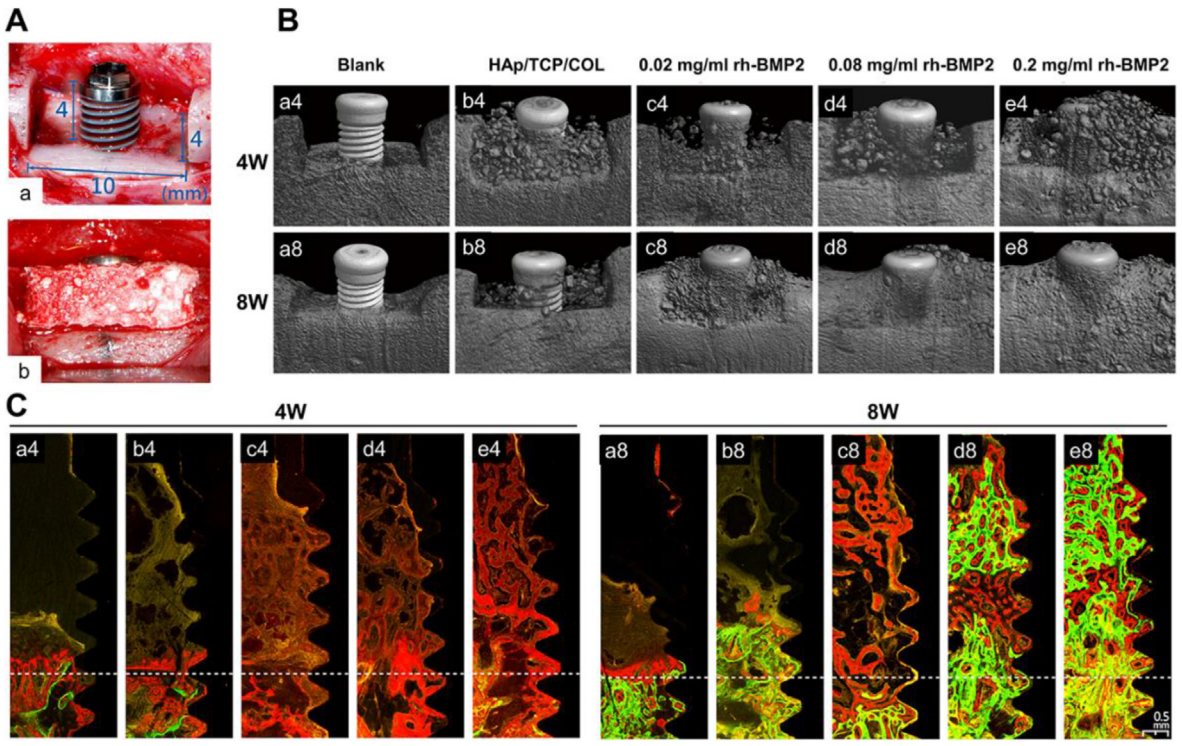


Fig. 5. (A) (a) Saddle-type bone defect with dental implant insertion. (b) Lateral view following the placement of HAp/TCP/Col composite and cover screw. (B) Representative 3D CT reconstruction. (C) Merged confocal microscope images of the two fluorochromes. Dotted line: original bone level [160]. Reproduced under the Creative Commons Attribution 4.0 International License [160]. Copyright 2021, John Wiley & Sons.

continuous release of BMP-2 derived peptides for 15 weeks, providing continuous stimulation for bone formation and reconstruction, and achieving similar osteogenic effects to rhBMP-2 [112].

4.3. Loading of drugs

Bisphosphonates (BPS), as a representative of osteoclast inhibitors, are commonly used drugs in clinical treatment of osteoporosis and metabolic bone diseases, and can reduce bone loss caused by unloading or stress shielding [172] and have been shown to possess strong osteogenic induction capability [173,174]. The collagen/hydroxyapatite (COL/HA) materials, which were loaded with alendronate (ALN), supported the adhesion and proliferation of MC3T3-E1 [175]. In the ectopic osteogenesis model, the adsorption of rhBMP-2 and zoledronic acid (ZA) by MC increased the bone volume by six times compared with rhBMP-2 alone. That is, ZA promoted the bone formation ability of rhBMP-2 [110] (Fig. 6). Compared with the direct adsorption loading strategy, the PLGA microspheres coated with ALN are incorporated into the MC during the preparation and the composite scaffold is soaked in BMP-2 solution to produce a dual agent delivery system. This offers a method to co-deliver synthetic and anti-decomposition drugs, realizing the sequential release of BMP-2 and ALN, and to fully exploit the synergistic effect of BMP-2 and ALN, significantly promoting bone regeneration [48].

Human parathyroid hormone-related peptide (osteostatin) can play a dual role in the stimulation of bone regeneration, promoting osteogenesis and suppressing osteoclastogenesis. Some researchers have loaded the pentaptide of parathyroid hormone (PThrP107-111) onto the COL/HA scaffold by chemical fixation. The composite scaffold has sustained peptide release *in vitro*. Functionalized scaffolds significantly promoted new bone formation [176]. In addition, collagen/poly (vinyl alcohol)/-propranolol/hydroxyapatite composite scaffold (CPPH), using a 3D printing technique *in vitro* gradual-release propranolol, indicated that the local adrenergic receptor blockers may promote bone defect repair by stimulating osteogenesis differentiation, inhibiting osteoclast formation, and increasing bone marrow mesenchymal stem cell migration [177]. Additionally, MC composite materials loaded with recombinant osteoprotegerin (OPG) can significantly reduce the maturation and resorption activity of primary osteoclasts. In a rabbit calvarial defect model, the defect area reconstructed with the composite material exhibited higher mineralization, hardness, and resistance to micro-fracture [178].

4.4. Doped inorganic metallic elements

Strontium has the dual effect of inhibiting bone resorption and promoting bone formation, and its doping in MC is a current research hotspot. Strontium promotes osteoblast differentiation by activating Ras/MAPK and Wnt/β-catenin signaling pathways and transcription factor Runx2 [179,180]. In addition, it interacts with membrane-bound calcium-sensing receptors [181] and affects osteoclast paracrine signaling [182]. Adding Sr into the hydroxyapatite (HA) altered its crystal lattice and resulted in a concentration-dependent inhibition of mineralization. However, the PILP method for synthesizing intrafibrillar minerals was not affected by Sr doping [183]. In the process of MC scaffold preparation, calcium is replaced by strontium to produce strontium-modified mineralized collagen [153], and composite materials can release strontium ions (Sr^{2+}), and the concentration of Sr^{2+} released by 50% and 100% strontium-substituted scaffolds is in a range that is conducive to the dual effect of strontium on bone metabolism [153]. Strontium-modified MC significantly promotes the proliferation and osteogenic differentiation of hBMSC. After being soaked in BMP-2 solution and implanted at the bone defect site, the cross-segmented bone bridge was achieved within 6 weeks. Strontium facilitated bone regeneration in a BMP-2-mediated femoral defect model in mice and improved the mechanical properties of the bridged defect. With the increase of strontium concentration, the quality of new bone tissue is significantly improved, the number of osteoblasts and blood vessels in the tissue increased, and the number of osteoclasts decreased, playing a dual role in regulating bone metabolism [154] (Fig. 7).

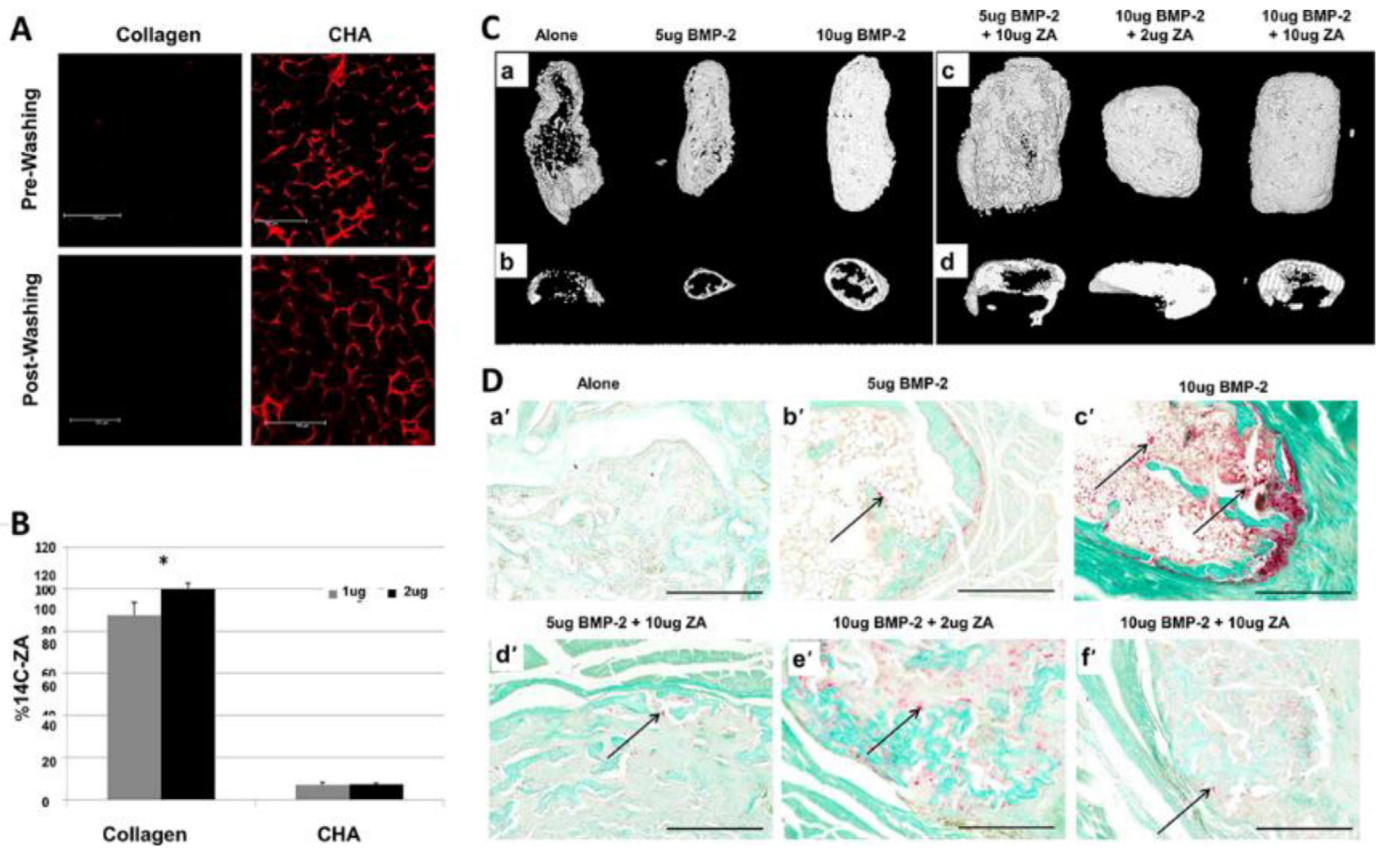


Fig. 6. (A) Representative images of the fluorescently labeled BP (Alexa Pam 647 – red color) within both scaffold types. (B) Quantification of ^{14}C-ZA elution from porous collagen and carbonated hydroxyapatite (CHA) scaffolds post-washing. *p < 0.01 in comparison to CHA elution of both 1 μg and 2 μg ^{14}C-ZA. (C) (a, b) Representative 3D CT reconstruction; (c, d) corresponding transaxial slices (stack of 50 slices) of μCT images of bone nodules resulting from each group. (D) Representative TRAP-stained histological sections of osteoclasts (stained in red) in trabecular-like structure of ectopic bone formed following 4 weeks of intramuscular implantation. The arrows indicate the stained osteoclasts. Scale bars = 500 μm [110]. Reproduced under the terms of the Creative Commons Attribution 4.0 International License [110]. Copyright 2014, Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Magnesium is one of the major ions in juvenile and nascent bone and tends to disappear in mature and aging bones [184,185]. The lack of magnesium ions in bone tissue can affect the morphology, size, and growth rate of crystals [156]. In the process of bone formation, Mg^{2+} can play a role in promoting the osteogenic differentiation of stem cells, promoting the adhesion and movement of osteoblasts, inhibiting osteoclasts, and regulating immunity [186], and indirectly influences bone mineral metabolism by inducing the production of nitric oxide to promote angiogenesis [187,188]. Magnesium-doped MC scaffolds simulate the composition and structure of human osteogenesis to a high degree, exhibit excellent biocompatibility [189], support the attachment and proliferation of hMSCs, and facilitate the formation of new bone and cartilage tissue [190]. In an ectopic bone model, a large amount of cancellous bone was produced within 2 weeks and gradually maturated, eventually forming new bone with lacunae and bone cells, showing faster and more efficient osteoinduction [158].

Various trace elements play a role in the process of bone regeneration *in vivo*, and MC doped with trace elements can significantly promote bone repair [191–193]. Zinc is an essential trace element, and a deficiency thereof affects bone development and is associated with the pathogenesis of osteoporosis [194,195]. Zn-doped HA/I-col material has a connected pore structure and continuous zinc ion release, which has excellent biocompatibility and can promote the osteogenic differentiation of rBMSCs and the repair of bone defects [191,192]. When adding zinc to the mineralized collagen-glycosaminoglycan precursor suspension, zinc-doped composites promote the growth and osteogenic differentiation of porcine ADscs [155]. Iron (Fe) and manganese (Mn) are essential trace elements in bone. Mn deficiency can weaken the activity of osteoblasts, leading to delayed osteogenic processes, bone deformation, growth inhibition, and even bone resorption [193]. Iron is a basic element in practically all organisms, which can promote reactive oxygen species production, and improve the antibacterial and osteogenic properties of iron-containing materials [196,197]. HIMC [152] incorporated with Fe^{2+} and Mn^{2+} significantly promote the adhesion, proliferation, and osteogenic differentiation of osteoblasts. Compared with pure HIMC scaffolds or commercial MC scaffolds, Fe/Mn composite HIMC scaffolds loaded with fresh bone marrow cells have better bone regeneration capability *in vivo*.

4.5. Additional elements

Fe^{2+}/Fe^{3+} can be incorporated into HA to obtain a new superparamagnetic phase with biocompatibility and bioabsorbability [3], namely FeHA nanoparticles. In one study, magnetic nanoparticles (MNPs) (such as Fe_3O_4 nanoparticles [198] and FeHA nanoparticles) were incorporated into MC to obtain an intelligent magnetic scaffold, which can apply mechanical stimulation to cells by external magnetic fields, and by adjusting the remote applied magnetic field, the mode and

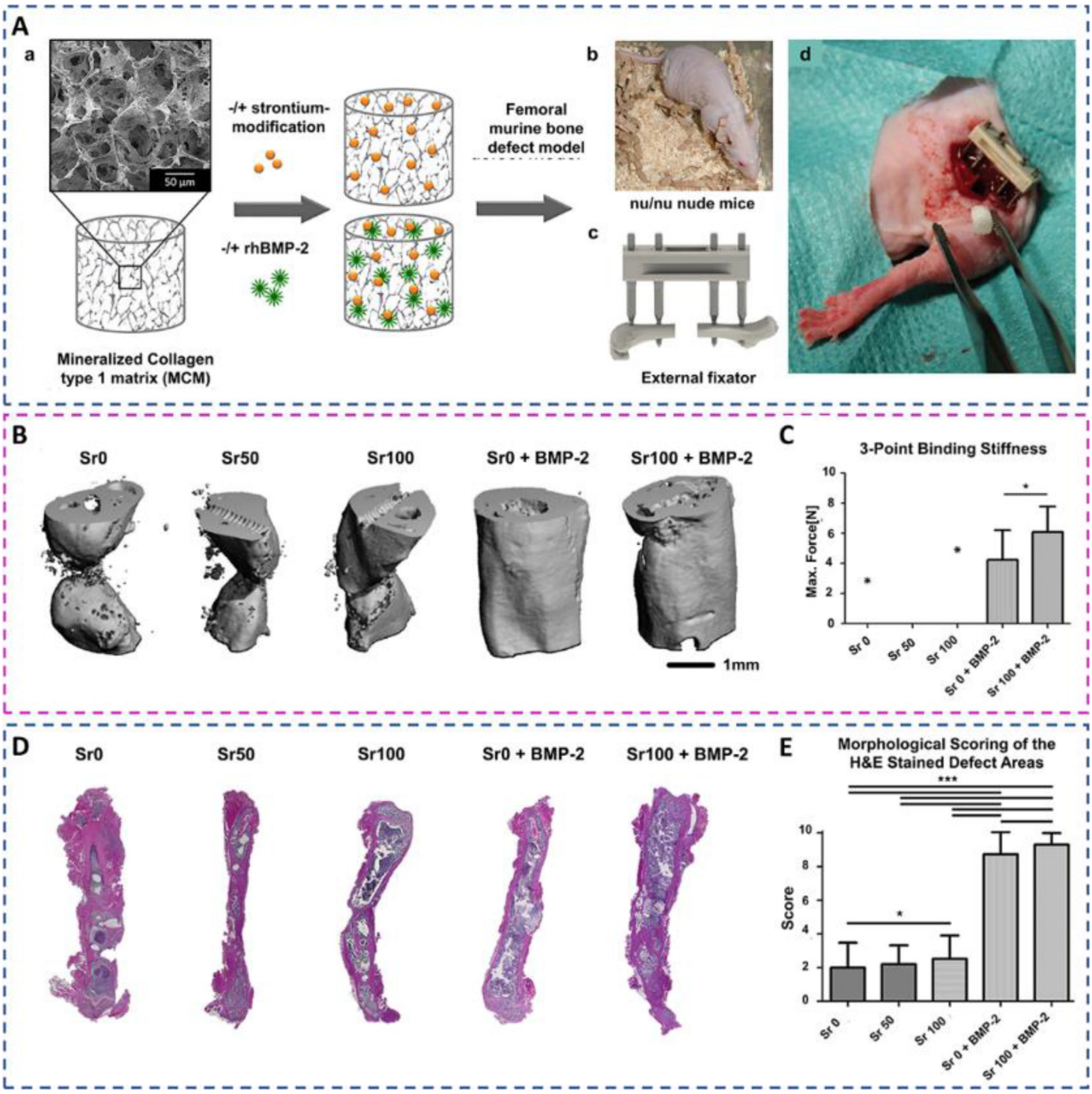


Fig. 7. (A) Experimental design *in vivo*. SEM image of a strontium-containing MC type I scaffold (a). The scaffolds were functionalized with and without rhBMP-2 and implanted into 2 mm bone defects of nude mice (b–d). The defects were stabilized by an external fixator (c). (B) μCT evaluation of the bone volume at the defect site. (C) The result of three-point binding stiffness. (D) Histological staining of femurs at 6 weeks after surgery. (E) Morphological scoring of the HE stained defect areas [154]. Reproduced under the Creative Commons Attribution License [154]. Copyright 2020, John Wiley & Sons.

intensity of mechanical stimulation for cells can be controllable. Studies have found that, compared with static magnetic fields, mechanical stimulation with periodic magnetic fields can better promote osteogenic differentiation of MSCs [3,198]. Furthermore, adding multi-walled carbon nanotubes (MWCNT) to MC can greatly enhance scaffold hardness (up to 10 times), promote bone marrow MSC proliferation and migration, enhance osteogenic differentiation, and significantly facilitate new bone formation [199]. Radially aligned MC fibers that incorporate nanosilicon (RA-MC/nSi) further promote the recruitment of host repair cells to the defect area in the skull, thereby facilitating bone regeneration [200].

5. Coordination of multifunctional MC

Bone induction and bone formation *in vivo* are complex and continuous processes and require the coordination of various sides, particularly for some complex bone defects. In addition to enhancing osteogenic function during the bone regeneration process, it is also important to coordinate with other functions such as angiogenesis, immunomodulation, and anti-infective properties to improve the bone microenvironment, thereby promoting regeneration and accelerating the healing processes (Table 3).

Table 3
Multifunctional coordination of MC.

Classification of MC	Synthetic strategy	Active factors	Loading strategy	Cells or animal models	Major findings	Reference
HIMC	PILP	Antimicrobial peptides	Immersing	hMSCs	Potent by contact killing of Gram-negative *Escherichia coli* and Gram-positive Streptococcus gordonii as well as cytocompatible to hMSCs.	[201]
EMC	CIMC	GAG	Mixing	hMSCs, HUVEC, THP-1	Directly and indirectly influence overall osteogenic potential and mineral biosynthesis as well as angiogenic potential and differentiation of monocyte.	[202]
EMC	CIMC	PL, HCM, ATE	Chemical bonding	hMSCs, HUVEC	Chemically attracted hMSCs and promote the prevascular structures formation.	[121]
EMC	CIMC	BMP-2, VEGF	Immersing	mandibular defect model in the rabbit	Improve angiogenesis and osteogenesis.	[108]
EMC	CIMC	rhBMP-2	Immersing	hMSCs, Femoral defect in the rabbit	Enhanced the osteogenic differentiation capacity of rBMSCs. Promote bone regeneration.	[111]
MC	DMA	Zinc Silicate	Mixing	BMSCs, critical size rodent calvarial defect model	Promotes BMSC migration, differentiation, and vessel formation.	[203]

PL, Platelet-rich plasma lysate; HCM, hypoxia-conditioned medium; ATE, adipose tissue extract; HUVEC, human umbilical vein endothelial cell; VNC, Vancomycin hydrochloride; GNT, gentamicin sulfate.

5.1. Synergy of angiogenesis and osteogenesis

VEGF is a specific GF acting on vascular endothelial cells, which can regulate the migration, proliferation, and capillary formation of endothelial cells [145,204]. It has been found that different patterns of VEGF release appear to affect angiogenesis in different ways. The continuous release of a tiny amount of VEGF from heparin-modified MC can affect the formation of vascular structures, such as stabilizing immature vascular structures, while the sudden release of VEGF accompanied by non-heparin-modified MC seems to be more conducive in tubule formation and sprouting [113]. VEGF was encapsulated in alginate microspheres, and then mixed into MC scaffolds, which could promote the formation of tubules by promoting vascular endothelial cells *in vitro*, and significantly promote angiogenesis and bone regeneration in a skull defect model [135]. In addition, non-sulfurized anionic glycosaminoglycan hyaluronic acid may act synergistically with VEGF to promote angiogenesis *in vivo* [205]. Based on the findings of the present study, artificial VEGF mimic peptides had the same function of promoting the angiogenic as VEGF proteins [206].

The concept that enhancing mature vascularized bone regeneration by coupling osteogenesis and angiogenesis is an essential concept of biomaterial modification, which can be exerted by the dual delivery of angiogenic and osteogenic factors [108,207]. MC loaded with low doses of BMP-2 and VEGF showed superior release kinetics *in vitro* [163], and lead to more rapid and effective bone regeneration *in vivo*; the volume of new bone increased about 28 times and the area of new bone increased about seven times compared with MC without growth factors [163]. In one study, an MC scaffold was used to incorporate CS microspheres loaded with RhBMP-2. The scaffolds were then immersed in a solution of VEGF to enable the sequential release of both factors, which facilitated bone growth. Through the synergistic and additive effects of the growth factors, VEGF promoted angiogenesis initially, followed by RhBMP-2, which led to bone formation [208] (Fig. 8).

The hypoxic conditioned medium (HCM) of hBMSC is a mixture of various signaling factors, has great potential to induce directional cell migration, and contains elevated concentrations of angiogenic factors, which can promote the formation of vascular structures [209]. HCM and alginate composite, as a central repository, integrate into the MC scaffold. The composite system was obtained after calcium crosslinking, which can slow the release signal factor and form a concentration gradient around the scaffolds, stimulating hBMSC directional migration to the center of the scaffolds, completing the recruitment of cells. Moreover, the composite system provides oxygen and nutrients for cells and accelerates the healing of bone defects by promoting angiogenesis [127]. In addition, heparin-modified MC scaffolds loaded with various concentrated mixtures of growth factors can not only play a role in promoting bone formation but also promote angiogenesis [121]. Silicate/nano-hydroxyapatite/collagen (ZS/HA/Col) scaffolds can create a favorable osteogenic microenvironment and regulate monocytes through the p38 MAPK pathway, promoting migration, differentiation of BMSCs, angiogenesis, and bone regeneration *in vivo* [203]. Additionally, some natural polymers (such as glycosaminoglycan, hyaluronic acid, and heparin) have excellent biological activity and biodegradability, and show enhanced osteogenic and angiogenic properties when combined with MC [47,202,210–213].

5.2. Synergy of immunomodulation and osteogenesis

After biomaterials are implanted in the human body, a series of immune responses develop, which not only determine the fate of the biomaterials but also has an impact on the results of bone regeneration [88]. Excessive immune responses cause fibrotic encapsulation, tissue destruction, and even implant-tissue separation, rejection, and other poor prognoses [214,215]. Macrophages are the dominant cell type involved in acute and chronic inflammation and the subsequent wound healing or fibrotic response. Activated macrophages exhibit M1 (proinflammation) and M2 (tissue repair) phenotypes. The switch and balance between M1 and M2 phenotypes are needed by tissue repair, and the polarized macrophages recruit other immune cells to inflammatory sites and activate the complement and adaptive immune system by secreting different cytokines and small molecules [216–218]. The Ding group has conducted a series of studies on the preparation of bone immune-regulating tissue engineering scaffolds. The composite scaffold has strong immune-regulating ability, significant angiogenic capacity, and strong osteogenic ability. In vivo, it significantly promotes bone defect repair [219,220]. Biomimetic collagen is an ideal bone repair material. Its nanostructure regulates the polarization of macrophages during bone regeneration and affects the outcome of bone regeneration [88,221] (Fig. 9).

Compared with pure collagen scaffolds, the expression of inflammatory and immune response genes associated with macrophages cultured on MC were downregulated. The expression of genes associated with cell proliferation, differentiation, tissue repair, and reconstruction were increased [38]. Researchers have found that the MC coating on the surface of titanium implants can regulate the phenotype of macrophages by triggering the integrin-related cascade pathway, thereby promoting osteogenic differentiation of mesenchymal stem cells [222]. Adrenaline affects macrophage polarization regulated by MC via the PI3K/Akt signaling pathway [38]. The surface roughness and the nanostructure of MC regulates the polarization of macrophages [223]. On the rough

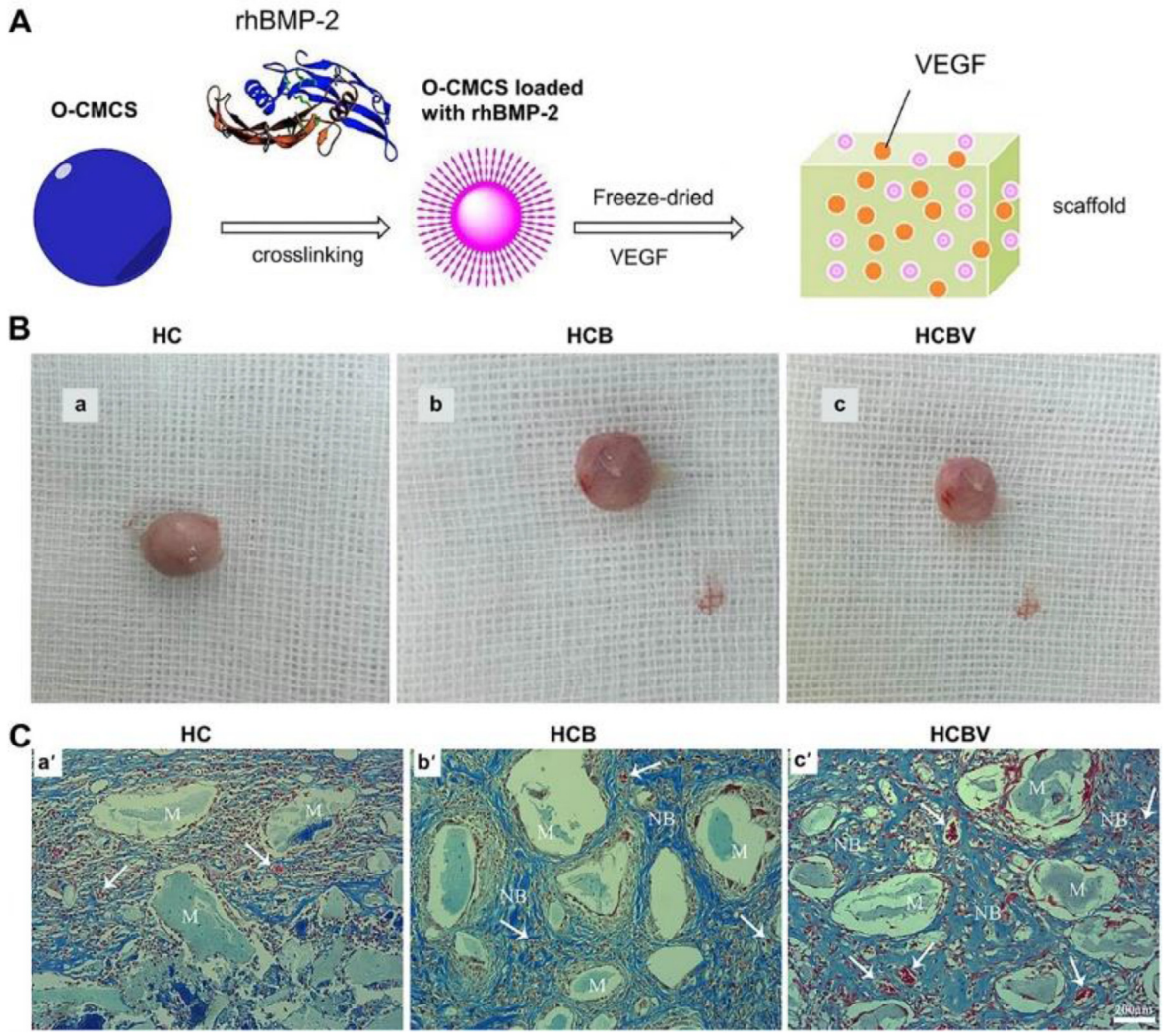


Fig. 8. (A) Simple method to prepare HA/COL composite. (B) Composites are implanted into animals for four weeks after removal and observed. (C) Masson staining images of each group, four weeks after implantation. NB, deposition of new bone; M, bone-filling material; arrows refer to the new blood vessels. Reproduced with permission [208]. Copyright 2019, Elsevier.

surface, macrophages tend to polarize into the inflammatory M1 phenotype and secrete inflammatory factors (tumor necrosis factor-α and interleukin-6) at high levels. While, on the smoother surface, macrophages induce polarization toward the M2 phenotype [223]. IMC and HIMC promote the polarization of M2 macrophages and increase the expression of M2-related anti-inflammatory cytokines IL-10 and arginase-1 at the cell, protein, and gene levels [31,88,221]. However, EMC mainly stimulates macrophages toward M1 polarization and expresses higher levels of the M1-related genes, including iNOS and IL-6 [31,88,224]. Other research has indicated that HIMC promotes M2 macrophage polarization though regulation of IL-4 expression and secretion, and the polarized macrophages strongly promote the osteogenic differentiation of MSCs and bone regeneration by secreting IL-4, but do not affect the recruitment process of MSC [31]. In addition, M2 macrophage-related small extracellular vesicles (sEVs) can promote the osteogenic differentiation of MSCs through the BMP2/Smad5 pathway. This increases the expression of osteoblast differentiation markers (BMP2, BGLAP, COL1, and OSX) and mineral deposition, and promotes endogenous bone regeneration [35].

5.3. Synergy between anti-infective and osteogenic

The treatment of infectious bone defects is an intractable problem to be solved in orthopedics. Systemic use of antibiotics is ineffective due to insufficient blood supply to the site of infection and increasing bacterial resistance [225]. For this reason, developing new versatile in situ bone defect repair scaffolds can effectively deal with infectious bone defects. Antibiotic-loaded MC (such as with antimicrobial peptides and some Chinese herbs) is a common strategy to achieve antibacterial function [201,226–228]. Directly mixing antibiotics with collagen or HA is the simplest method to prepare MC with antibacterial properties, which can achieve the effective release of active vancomycin [229]. The COL/HA electrospun fiber membrane is loaded with vancomycin and gentamicin by soaking or immersion [230], which sustains the release of drugs with antibacterial activity in high concentrations. The combined application of the two drugs has a complementary effect, and is more effective than a single administration of either agent. Furthermore, it is devoid of cellular toxicity. A gentamicin/HA/collagen bone nanocomposite is an excellent bioabsorbable anti-infective bone cavity filler without cytotoxicity, and can be used for the prevention of initial infection [231].

The adsorption, activity, and release of antibiotics on MC are affected by multiple factors. Magnesium modified mineralized collagen has excellent osteogenic properties, and antibiotics (such as vancomycin and gentamicin) loaded into magnesium modified MC composite materials by soaking or immersion were developed to improve the osteogenic and anti-infection properties. The magnesium doping leads to greater drug

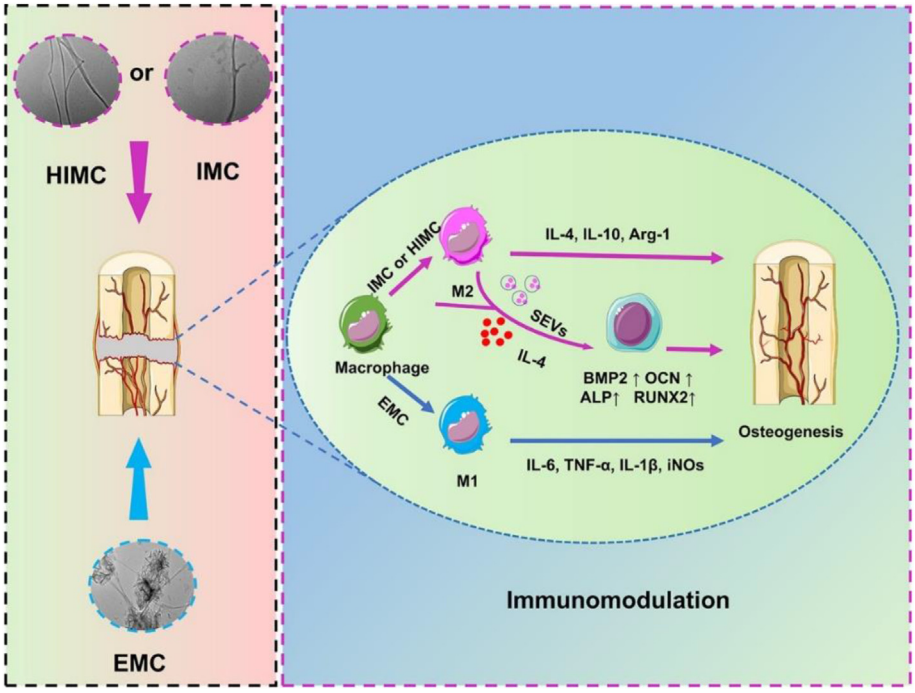


Fig. 9. Potential molecular mechanism of how macrophage polarization activated by MC with different nanostructures affects the process of endogenous bone regeneration.

retention but does not affect the antibacterial activity of the drugs [227]. The binding mode of vancomycin and COL/HA material does, however, influence the drug's activity. Compared with directly incorporating vancomycin into the COL/HA electrospun liquid, vancomycin loaded COL/HA after electrospinning shows stronger antibacterial activity [147]. However, HA modification in MC does not negatively affect the sustained release of vancomycin [229] or initial release thereof [147, 230]. In addition, various antibiotics have different adsorption capacities upon MC. The superior adsorption capacity of antibiotics onto MC results in more effective activity compared to antibiotics with lower adsorbability, leading to improved therapeutic outcomes in murine models of acute osteomyelitis [107] (Fig. 10).

Silver ions exhibit excellent antibacterial properties by destroying bacterial cell membranes and binding microbial DNA and sulfhydryl groups of metabolic enzymes, thereby directly incorporating silver ions into various natural and synthetic polymers to optimize the antibacterial effect of materials [232–234]. In one study, silver nanoparticles were prepared by the interaction between tannic acid and silver nitrate, and then incorporated into a collagen matrix [235]. The composite was then immersed in simulated body fluid. Lastly, a composite MC loaded with silver nanoparticles was obtained though collagen self-assembly and mineralization. The composite exhibited good mechanical performance and antibacterial activities, and showed perfect biocompatibility to MG-63 cells and red blood cells [235]. In another study, silver-doped

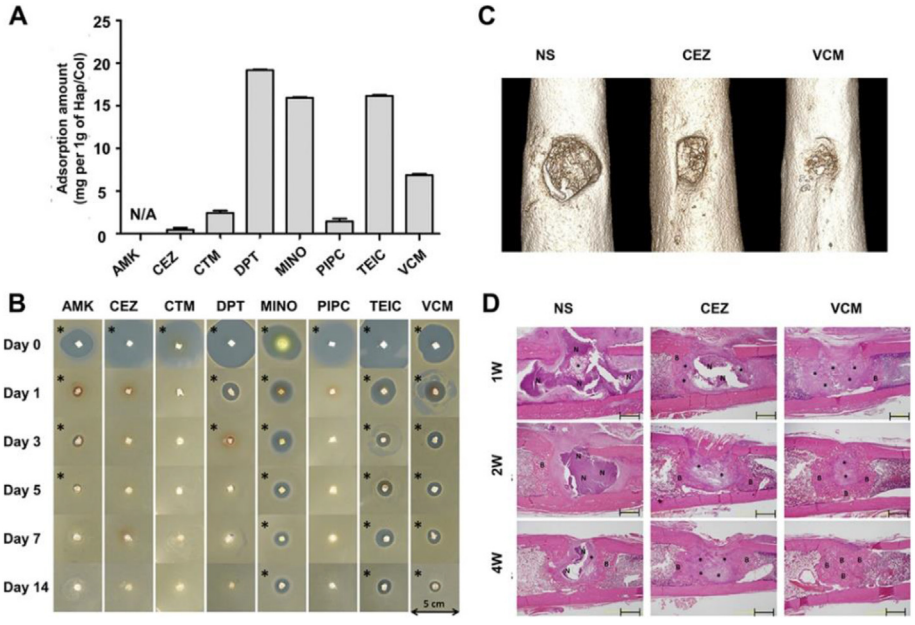


Fig. 10. (A) The amount of adsorbed antibiotic (mg) per 1 g of HAp/Col. N/A, not available. (B) Representative photographs of culture dishes. The translucent circles are inhibitory zones. *Effective inhibitory zone. (C) 3D CT reconstruction of bone holes at 4 weeks after implantation. (D) Hematoxylin and eosin staining images of the implant site at different times after implantation (scale bar: 1000 μm) [107]. Reproduced with permission [107]. Copyright 2019, the Authors. Published by Wiley Periodicals.

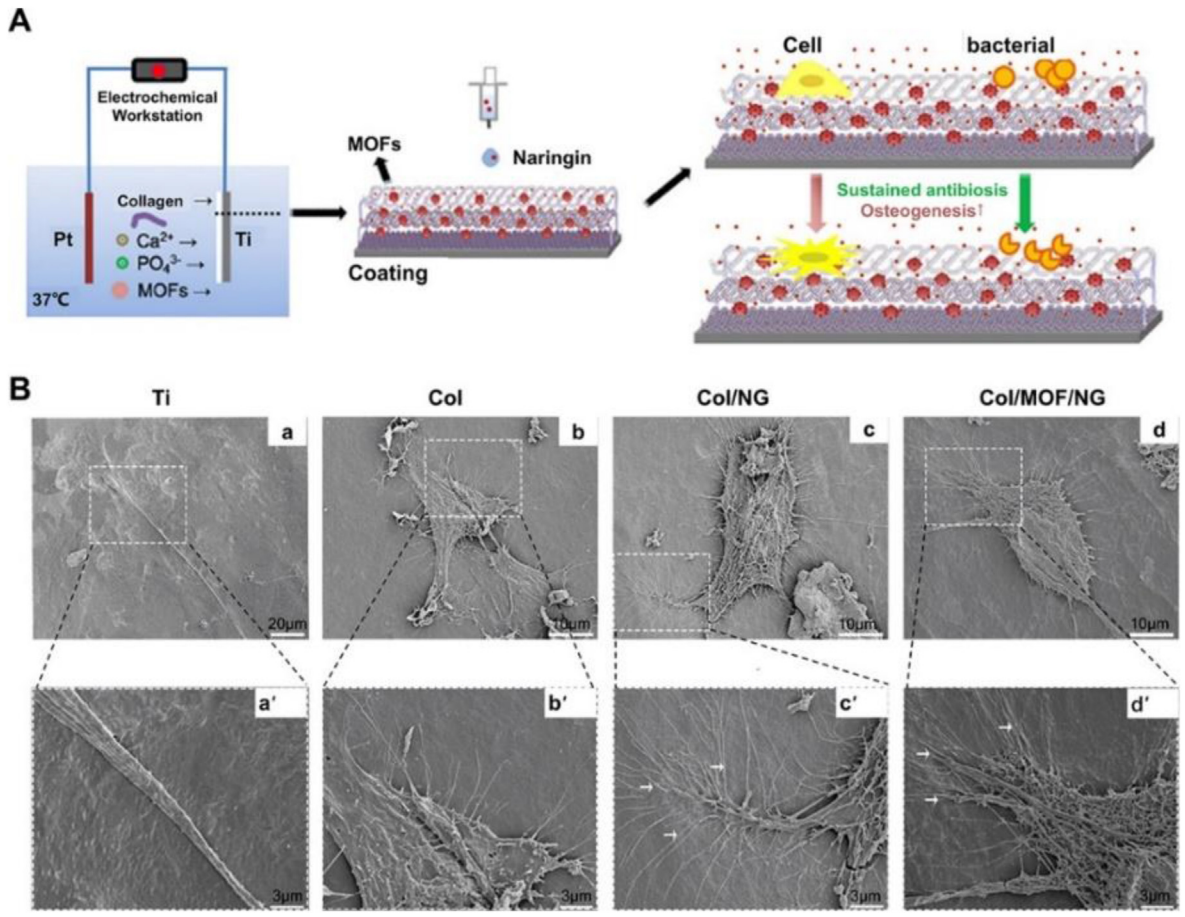


Fig. 11. (A) Schematic illustration of the MC coating on titanium with the aid of metal-organic framework nanocrystals to control the release of naringin, which could enhance osseointegration and simultaneously inhibit microbial cell growth. (B) Morphological observation of MSCs on various substrates. Filopodia are indicated by white arrows [226]. Reproduced under the terms of the Creative Commons Attribution License [226]. Copyright 2017, American Chemical Society.

hydroxyapatite (AgHAp) was prepared by the co-precipitation method and then added to a collagen gel. Silver-doped MC was obtained after freeze-drying. This composite also has strong antibacterial properties, and the inhibition zone increased sharply with the increase in the silver concentration [236]. In addition, graphene oxide (GO) [237], antimicrobial peptide GL13K [201], and naringin [226] were introduced into mineralized collagen materials to obtain modified MC composites, which have excellent biocompatibility, enhanced osteogenic properties, and anti-infection effects (Fig. 11).

6. Discussion

MC has attracted considerable attention because of the similarity to the chemical composition and/or nanostructure of natural bone tissue, and has been evaluated as a relatively common scaffold material in bone tissue engineering. There are currently many commercially available MC products that have been approved by regulatory agencies for clinical use and have demonstrated superior biological properties in bone defect repairs and regeneration. However, for complex bone defects, MC exhibits insufficient osteoinduction and poor antibacterial properties. As is well known, due to the loose and porous structure, large surface area, and inclusion of nano-HA, MC is suitable as an effective carrier for cells, various AFs, and drugs. Moreover, calcium ions in MC can be doped and replaced by various inorganic metallic ions, and impart enhanced osteogenesis, angiogenesis, immunomodulatory, and anti-infection properties to MC. The method to load AFs on MC is important and can be categorized into four categories, including the adsorption onto MC by immersing, binding to MC after surface modification, mixing with raw materials of MC, and polymer encapsulate. Therefore, the efficient and orderly release of single or multiple AFs can be achieved by using different loading methods or the combination of different loading methods, to realize the multi-functionalization of MC. In the process, attention should be paid to the influence of physical and chemical conditions on the biological activity of AFs and the influence of AFs on the degradation performance of MC, crystal morphology, nanostructures, and content of HA. When loading multiple AFs, the interaction between different factors should be noted, and this may affect the release curve and bioactivity. In the process of in situ bone tissue regeneration, functionalized MC not only acts as a support material, but also as a drug delivery system to continuously deliver AFs locally, to exert an anti-infection, immunomodulatory, angiogenesis, and osteogenesis function, and finally facilitate bone regeneration and repair various complex bone defects.

In recent years, despite the impressive progress in the study of functionalized MC materials, numerous problems remain to be solved. IMC and HIMC have excellent mechanical and biological properties compared to conventional MC. However, the biomimetic preparation process *in vitro* is complex, time-consuming, involves numerous variables, and a unified preparation strategy has not yet been formed, which is not favorable for the industrial production and uniform results between different studies. In addition, present research is at the stage of preparing MC with a level-2 hierarchical structure in the multilevel layered structure of bone tissue. Achieving biomimicry at higher levels of the layered structure of bone tissue becomes a formidable challenge. Strengthening the knowledge of the biomineralization process *in vivo*, and a precise understanding of the nature and function of collagen type I and numerous

non-collagenous proteins, as well as continuously striving to improve biomimetic preparation strategies *in vitro*, will contribute to the successful fabrication of MC with the more intricate hierarchical structure of natural bone. MC with different nanostructures have different biological properties, and the precise mechanisms of pro-osteogenic effects involve a multitude of aspects, such as osteogenesis, angiogenesis, and osteoimmunomodulation. Future research should focus on two important areas. First, the development of more advanced biomimetic MC preparation methods. Second, the direction of future research should focus on encapsulating and precisely controlling the release of AFs using a combination of multiple AFs and loading strategies that can function collaboratively. Ultimately, the preparation of a functional MC composite system that can effectively repair bone defects of varying degrees would be a crucial consideration for future studies. We believe that by further investigating mineralization mechanisms *in vivo*, improving the preparation strategies for biomimetic MC *in vitro*, and introducing advanced drug sustained release systems, which are constantly optimized with the release of AFs, the development of a functional MC to meet the diverse needs of patients and address clinical requirements is well within reach.

Data statement

No data was used for the research described in the article.

Author contributions

Xiujie Zhu: Investigation, Data curation, Conceptualization, Methodology, Writing – original draft. Chenyu Wang: Method-ology, Validation, Formal analysis, Resources, Writing – review & editing. Haotian Bai: Validation, Resources, Writing – review & editing. Jiaxin Zhang: Writing – review & editing, Supervision. Zhonghan Wang: Writing – review & editing. Zuhao Li: Supervision, Conceptualization, Validation, Writing – review & editing. Xin Zhao: Writing – review & editing. Jincheng Wang: Writing – review & editing. He Liu: Writing – review & editing, Supervision, Funding acquisition.

Role of the funding source

This work was financially supported by the National Natural Science Foundation of China (Grant Nos. 82001971, 82102358 and 82202698); Scientific Development Program of Jilin Province (Grant Nos. 20200403088SF, 20220204117YY, YDZJ202201ZYTS086, 20200404202YY and 20200802008 GH); Program of Jilin Provincial Health Department (Grant No. 2020SC2T064 and 2020SC2T065); Project of "Medical + X″ Interdisciplinary Innovation Team of Norman Bethune Health Science Center of Jilin University (Grant No. 2022JBGS06); China Postdoctoral Science Foundation (Grant No. 2021M701384); Bethune Plan of Jilin University (Grant No. 2022B27). Postgraduate innovation Research program of Jilin University (Grant No. 4190214223Q0)

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.

Data availability

No data was used for the research described in the article.

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