Biomaterials Translational

Hydrogel microspheres for bone regeneration through regulation of the regenerative microenvironment

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Key Words:
bone regeneration; bone tissue engineering; hydrogel microspheres; regeneration microenvironment

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ABSTRACT

Bone defects are a prevalent category of skeletal tissue disorders in clinical practice, with a range of pathogenic factors and frequently suboptimal clinical treatment effects. In bone regeneration of bone defects, the bone regeneration microenvironment—composed of physiological, chemical, and physical components—is the core element that dynamically coordinates to promote bone regeneration. In recent years, medical biomaterials with bioactivity and functional tunability have been widely researched upon and applied in the fields of tissue replacement/regeneration, and remodelling of organ structure and function. The biomaterial treatment system based on the comprehensive regulation strategy of bone regeneration microenvironment is expected to solve the clinical problem of bone defect. Hydrogel microspheres (HMS) possess a highly specific surface area and porosity, an easily adjustable physical structure, and high encapsulation efficiency for drugs and stem cells. They can serve as highly efficient carriers for bioactive factors, gene agents, and stem cells, showing potential advantages in the comprehensive regulation of bone regeneration microenvironment to enhance bone regeneration. This review aims to clarify the components of the bone regeneration microenvironment, the application of HMS in bone regeneration, and the associated mechanisms. It also discusses various preparation materials and methods of HMS and their applications in bone tissue engineering. Furthermore, it elaborates on the relevant mechanisms by which HMS regulates the physiological, chemical, and physical microenvironment in bone regeneration to achieve bone regeneration. Finally, we discuss the future prospects of the HMS system application for comprehensive regulation of bone regeneration microenvironment, to provide novel perspectives for the research and application of HMS in the bone tissue engineering field.

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http://doi.org/10.12336/biomatertransl.2024.03.002

How to cite this article:
Zhang, P.; Qin, Q.; Cao, X.; Xiang, H.; Feng, D.; Wusiman, D.; Li, Y. Hydrogel microspheres for bone regeneration through regulation of the regenerative microenvironment. *Biomater Transl.* **2024**, *5*(3), 205-235.

Introduction

Bone is highly mineralised connective tissue, essential for structuring complete human anatomy, regulating blood cell regeneration and osteocyte physiological functions, and maintaining calcium-phosphorus metabolism. It is a vital organ that supports body movement, protects the internal organs, exerts haematopoietic function, and participates in the regulation of the endocrine system.[1, 2] Bone defect refers to the loss of skeletal tissue due to trauma, infection, tumour removal, or developmental abnormalities. When the bone defect is more than 1.5-times the diameter of the backbone, it exceeds the maximum ability of the bone to heal by itself, which is called a critical bone defect.[3] It is estimated that yearly, more than 1.5 million patients worldwide undergo

bone grafting surgery to treat bone defects yearly, with a corresponding medical cost of approximately $1.5 billion.[1,4] In addition to affecting the quality of life of individuals because of the limited mobility and pain, the treatment of bone defects imposes a substantial economic burden on society. Therefore, further research and exploration of effective strategies for the prevention, diagnosis, and treatment of bone defects is essential for global health. In a state of bone defect, the body will initiate the self-repair process of bone tissue, which requires the coordination of multiple functional components. These functional components, benefit the bone regeneration process, and jointly build the bone regeneration microenvironment that includes physiological, chemical, and physical components. The physiological microenvironment supports bone regeneration through multiple cellular and intercellular signalling crosstalk, which promotes vascularised osteogenesis, immune-regulated bone metabolism, neurogenic osteogenesis, and other osteogenic processes. In the chemical microenvironment, oxygen and pH can also regulate cellular energy metabolism and physiological functions. Meanwhile, cytokines such as vascular endothelial growth factor (VEGF), neurogenic trophic factor (NGF), and bone morphogenetic protein-2 (BMP-2) released by immune cells, bone marrow mesenchymal stem cells (BMSCs), and other cells can act on endothelial cells, neurocytes, osteocytes, and other target cells, thereby regulating the proliferation, differentiation, migration, apoptosis, and other physiological processes of these target cells, to jointly enable the regulation of the chemical microenvironment, and promote bone regeneration. In addition, physical stimuli such as mechanical, photo, thermal, electrical, magnetic, and acoustic constitute the physical microenvironment of bone regeneration, and these stimuli regulate osteoblast behaviour and the differentiation potential to promote bone regeneration.[5] Therefore, the microenvironment of bone regeneration is a crucial and integral aspect for the regulation of bone regeneration, and therapeutic strategies based on the comprehensive regulation of bone regeneration microenvironment is a critical concept to guide the design of bone regeneration biomaterials.

Clinical treatments for bone defects include autologous, allogeneic, and artificial bone graftiing. Compared to allograft, autologous bone is still the 'gold standard' for the clinical treatment of bone defects because of its high biocompatibility and superior osteogenic properties.[6] Artificial bone grafting materials are frequently employed in clinical treatments. Zhang et al.[7] analysed 36 patients with femoral head necrosis over a period of 45 months. Among these patients, 18 chose bioceramic (β-tricalcium phosphate) bone grafting, while the remaining opted for autologous bone grafting. The analysis revealed that the Harris score in the β-tricalcium phosphate implant group was 64.22%, indicating that the bioceramic grafting material offers advantages such as reduced trauma and a faster recovery of postoperative function. Resende et al.[8] conducted a study on 30 volunteers who underwent implant placement within 3 months following tooth extraction. In the experimental group, 15 patients who received nanohydroxyapatite implants exhibited implant calcium and phosphorus levels of 40.13% and 18.50%, respectively. In contrast, the control group, which received bovine xenografts, showed calcium and phosphorus levels of 37.50% and 17.30%, respectively. These results suggest that nanohydroxyapatite possesses superior osteogenic properties. However, autologous grafts have limitations such as scarcity of donor bone sources and a tendency to induce postoperative complications in the bone removal area, which limit its wide application in clinical treatment.[9] Many types of artificial bone grafting materials are available, such as hydroxyapatite, bioactive glass, and calcium-phosphorus bone cement. However, these artificial bone grafting materials cannot regulate the bone regeneration microenvironment comprehensively, and immune rejection may occur, hence, these biomaterials need to be further optimised.[10] To compensate for the poor bioactivity of artificial bone grafting materials, highly bioactive bone regeneration biomaterials that can enable the replacement of autologous bone tissue have become the focus of recent research.

Hydrogels, one of the most widely used biomaterials for bone tissue engineering, are cross-linked polymers formed from hydrophilic monomers with excellent bioactivity, degradability, high hydration, and solubility.[11] To expand the application scope of hydrogels in the medical field, researchers prepared hydrogels into spherical particles named hydrogel microspheres (HMS), ranging from 1 to 1000 μm, through batch emulsification, microfluidic emulsification, photolithography, and electrospray.[12] Due to its high specific surface area, high porosity, excellent encapsulation, and highly adjustable physicochemical properties, HMS can efficiently carry small molecule drugs, biological factors, stem cells, and other therapeutic components to the lesion site, and achieve controllable release, to accurately and effectively regulate treatment. Therefore, HMS has demonstrated broad potential applications in the field of bone tissue engineering. HMS can disperse excessive stress transmission through the relative movement between microspheres, thus reducing the negative impact of the shear force generated by injection on its loaded biological factors/drugs. Thus, it is widely used in the local treatment of osteoarthritis (OA).[13] In addition, through HMS carrier design, targeted delivery and controllable drug release can be achieved so that the drug can act on tumour cells more accurately and stably while reducing the damage to surrounding normal tissues.[14] This strategy is often applied to local drug delivery of bone tumours. In the field of bone regeneration, HMS is also regarded as an excellent biomaterial that can promote bone regeneration by regulating the physiological, chemical, and physical microenvironment. Therefore, HMS has fantastic potential to comprehensively regulate the microenvironment of bone regeneration.[12]

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This review focuses on the bone regeneration microenvironment and the application and mechanism of HMS in regulating it. We first introduced the interaction mechanism of various components in the bone regeneration microenvironment and its influence on the process of bone tissue regeneration and then introduced the preparation materials, methods, and functional characteristics of the system of HMS, as well as the application of HMS in bone tissue engineering. Furthermore, given the great potential of HMS in promoting bone tissue regeneration, we focused on the principle that the HMS system regulates the components of the bone regeneration microenvironment to achieve bone regeneration and explored the related molecular mechanisms that affect the bone regeneration process by affecting the cell physiological functions, chemical signals, physical stimuli, and other factors in the microenvironment. Finally, we reviewed the application prospects of the HMS system in the field of bone regeneration. Therefore, the aim of this review is to provide new ideas and inspiration for bone tissue engineering and regenerative medicine research.

We conducted a literature search using the terms 'bone regeneration', 'regeneration microenvironment', 'bone tissue engineering', and 'hydrogel microspheres' across PubMed, Web of Science, and Wiley databases for articles published between 2000 and 2024. In cases where the literature covered similar topics, preference was given to publications in the most recent or more authoritative journals.

Bone Regeneration Microenvironment

Bone regeneration microenvironment refers to the appropriate conditions created to promote bone tissue repair and regeneration after bone defects caused by trauma, infection, tumour, and other factors.[5] The bone regeneration microenvironment is composed of three key components: physiological microenvironment, chemical microenvironment, and physical microenvironment. Understanding the influencing factors and their mechanisms in the bone regeneration microenvironment can help to comprehensively regulate it, thereby accelerating the process of bone regeneration (**Figure 1**).

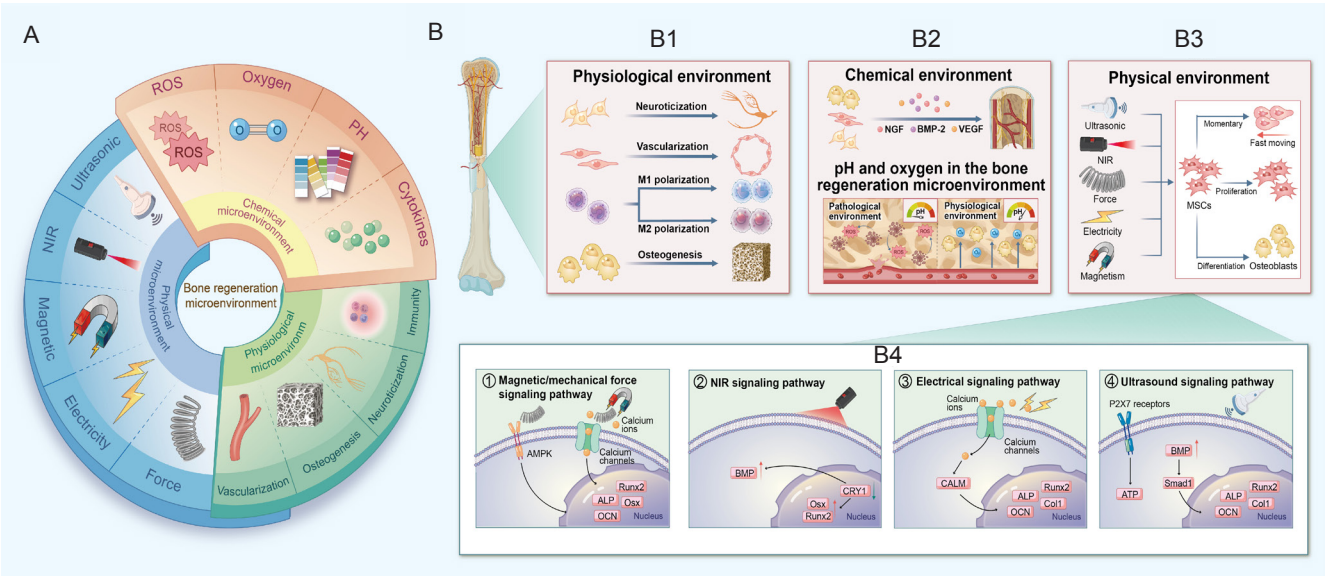

Figure 1. Schematic diagram of the bone regeneration microenvironment. (A) Physiological, chemical, and physical factors in the bone regeneration microenvironment; (B) Brief schematic diagram of the osteogenic process in the bone regeneration microenvironment. (B1) Physiological microenvironment in which neurons, endothelial cells, macrophages, and osteoblasts differentiate into neural tissue, vascular tissue, M1/M2 cells, and bone tissue. (B2) Chemical microenvironment in which cytokines such as NGF, BMP-2, and VEGF promote differentiation of neuronal cells, osteoblasts, and endothelial cells to jointly promote bone regeneration; pH and ROS of the microenvironments in pathological and physiological conditions. (B3) Physical microenvironments in which physical stimuli such as ultrasound, infrared light, mechanical force, electrical stimulation, and magnetism promote the migration, proliferation, and differentiation of MSCs into bone. (B4) Stimuli such as electrical stimulation, magnetic and mechanical forces, near-infrared light, and ultrasound promote osteogenesis expressed by osteocytes. Created with BioRender.com. ALP: alkaline phosphatase; AMPK: adenosine monophosphate-activated protein kinase; ATP: adenosine triphosphate; BMP-2: bone morphogenetic protein-2; CALM: calmodulin; Col1: collagen I; CRY: cryptochrome; MSC: mesenchymal stem cell; NGF: nerve growth factor; NIR: near infrared; OCN: osteocalcin; Osx: osterix; pH: potential of hydrogen; ROS: reactive oxygen species; Runx2: Runt-related transcription factor 2; Smad1: small mother against decapentaplegic homolog 1; VEGF: vascular endothelial growth factor.

Physiological microenvironment

The physiological microenvironment of bone regeneration includes the circulatory microenvironment, immune microenvironment, neuronal cell microenvironment, and osteoblastic microenvironment. In the circulatory microenvironment, neovascularisation leads to regeneration and provides oxygen and nutrients to the bone tissue and promotes bone regeneration by regulating osteoblast behaviour through haemodynamics. Meanwhile, in the immune microenvironment, various immune cells such as macrophages and neutrophils participate in the regulation of the inflammatory response and regulate the metabolic process of osteocytes by releasing cytokines and chemokines. In addition, the nervous system is also involved in regulating osteocyte metabolism, and central and peripheral nerve cells release neurotransmitters and growth factors to regulate osteoblast proliferation and differentiation, maintain bone tissue homeostasis, and repair damaged bone tissues.[15] In addition, osteoblasts are involved in the direct regulation of bone regeneration. Functionalised osteoblasts in bone tissue, including osteoblasts and osteoclasts, are involved in the synthesis of new bone matrix or resorption of old bone matrix through different metabolic directions and are directly involved in the process of bone tissue repair.

Circulatory microenvironment

Bone tissue regeneration and repair require good blood circulation support, and in the circulatory microenvironment of bone regeneration, vascular neovascularisation and haemodynamic regulation are crucial. Nutrients along with, oxygen, calcium, and phosphorus ions are transported from the vasculature to the diseased part of the body to participate in promoting the formation and mineralisation of the osteogenic matrix. There are two subtypes of capillaries in the epiphyseal and marrow cavities, namely the H- and L-type vessels. H-type vessels are mainly located in the epiphyseal region and subperiosteum and have high expression of proteins such as CD31 and endothelial mucin, whereas L-type vessels are mainly located in the metaphyseal region and have low expression of CD31 or endothelial mucin proteins.[16] Moreover, > 82% Runt-related transcription factor 2 (Runx2)-positive and 70% Osterix-positive bone progenitor cells were selectively localised around H-type endothelium. The H-type vascular endothelial cells secrete a variety of factors such as Noggin and VEGF that stimulate the proliferation and differentiation of bone progenitor cells, thereby regulating osteogenesis.[17] In addition, proper haemodynamics is essential for the regeneration and repair of bone tissue. Osteocytes are subjected to fluid shear forces from blood flow dynamics. The flow of blood through the skeletal vasculature generates shear forces on the surrounding osteocytes owing to the friction between the blood flow and surface of the osteocytes. Fluid shear acts as a mechanical stimulus that can affect osteocyte function and metabolism. Studies have shown that Notch 1 ligand Delta-like 4 (*Dll4*) mRNA expression in human mesenchymal stem cells (MSCs) is increased upon exposure to low fluid shear stress, suggesting that Notch signalling is involved in mechanically regulated osteogenic differentiation.[18] Vascular fluid shear and vascular pressure produce stimuli in BMSCs that lead to the activation of downstream biochemical signals through cellularly indicated integrin and Piezo1 ion channels, respectively, which in turn lead to the dephosphorylation and nuclear translocation of the intracellular transcription factors yes associated protein 1 and nuclear factor of activated T cells 2. Activated yes associated protein 1 binds to nuclear factor of activated T cells 2 and enhances transcriptional activity to more effectively promote the osteogenic differentiation of BMSCs.[19]

Immune microenvironment

During bone regeneration, the early inflammatory response to bone defects helps to clear necrotic tissue and cellular waste, and immune cells such as macrophages can provide healthy repair areas for subsequent bone regeneration by phagocytosis and degradation of these substances. In addition to regulating the inflammatory response, the immune system is also involved in aspects of osteocyte metabolism and neovascularisation that promote bone tissue regeneration and repair. Excessive inflammatory response may have negative effects during bone regeneration, and immune regulation can control the degree and duration of inflammatory response, maintain a moderate inflammatory response, and avoid excessive damage to bone tissue. For example, in an inflammatory environment, tumour necrosis factor-α produced by immune cells can promote lymphocyte and neutrophil apoptosis through SMAD signalling pathway to inhibit further inflammation and achieve immune regulation of inflammation.[20] Meanwhile, cytokines such as transforming growth factor-β (TGF-β) and BMPs produced by macrophages can promote the differentiation and activation of osteoblasts, activate osteoclast proliferation and bone matrix formation, and provide cellular basic conditions for bone tissue regeneration.[21] In addition, immune cells such as macrophages and T cells can release cytokines such as VEGF to promote the formation of new blood vessels.[22]

The activation of immune cells and secreted cytokines play an important role. In the early stage of fracture, the cells acting in the bone regeneration microenvironment are mainly neutrophils, polarised M1 macrophages, and early activated lymphocytes regulating bone metabolism. In contrast, in the late stage of bone regeneration, M2 macrophages are involved in the inflammatory regulation of the bone regeneration microenvironment and bone remodelling. After damage to bone tissue, neutrophils are activated and accumulate in the damaged area, releasing inflammatory mediators such as interleukin (IL)-1β and IL-6, which not only regulate the activation and proliferation of immune cells but also promote inflammatory responses.[23] In addition to neutrophils, T-helper 17 cells such as T helper cells in the T lymphocyte subset produce inflammatory factors like IL-17 and further contribute to the inflammatory response by releasing these inflammatory factors during bone regeneration.[24] Meanwhile, neutrophils modulate immune cell-mediated inflammatory responses by releasing reactive oxygen species (ROS) to impair mitochondrial function and trigger apoptosis-related responses, thereby regulating the balance between immune cell proliferation and apoptosis.[25] Meanwhile, macrophages derived from mononuclear phagocytes usually act as M1

and M2 types in bone regeneration. The balance between these two macrophage phenotypes is crucial during bone regeneration. M1 macrophages are mainly involved in the initial inflammatory phase. They release pro-inflammatory cytokines such as tumour necrosis factor-α, IL-1β, and IL-6, as well as oxygen free radicals and nitric oxide, which recruit other immune cells and stimulate an inflammatory response.[26] In addition, the activation of M1 macrophages inhibits bone formation and suppresses the process of bone regeneration by inhibiting the proliferation and differentiation of osteoblasts and promoting the activity of osteoclasts. M2 macrophages play a key role in the later stages of the healing process. They produce cytokines such as IL-10 and TGF-β to help induce osteoblast proliferation and differentiation and finally promote bone tissue repair.[27] Dendritic cells are specialised antigen-presenting cells of the immune system, most of which are generated from bone marrow and undergo a series of differentiation and maturation steps to form mature dendritic cells. They can capture cytokines, proteins, and damaged tissue debris released during bone regeneration and present these antigens to activate immune cells like T and B lymphocytes that are involved in the regulation of immune cell-to-cell interactions. Dendritic cells regulate the type and extent of the inflammatory response by releasing different types of cytokines and mediators, while dendritic cells may be anti-inflammatory by removing over-expressing inflammatory cells through 'cytoburial effects'.[28] Dendritic cells may also directly regulate bone regeneration by influencing osteoblast and osteoclast activity. Although the mechanisms and pathways involved have not been fully elucidated, the regulatory role of dendritic cells is essential for maintaining homeostasis in the bone regeneration process.[29]

Nerve cell microenvironment

In the bone regeneration microenvironment, the nervous system plays an important role in bone tissue repair and regeneration through the regulation of peripheral nerves and the central nervous system (CNS).

CNS regulation of osteogenesis: The CNS can not only release brain-derived neurotrophic factor (BDNF), exosomes, and other biologically active factors that are directly involved in the regulation of bone regeneration but also regulate bone metabolism through the neuroendocrine system, and the proposal of such a neuroendocrine axis reveals additional mechanisms of neuralised bone regeneration.[30] Through the hypothalamic-pituitary-target gland axis, endocrine signalling is integrated to regulate a variety of physiological processes including bone homeostasis. Growth hormone regulates protein synthesis mechanisms in osteoblasts by binding to the growth hormone receptor and activating downstream signalling pathways within the receptor, such as the Janus kinase-signal transducers and activators of transcriptionSTAT pathway.[31] These proteins may include collagen and bone matrix proteins, which are essential for bone tissue formation and repair. BDNF produced by the CNS supports neuronal survival, development, and functional maintenance, and participates in the regulation of osteoblasts. BDNF promotes bone regeneration by promoting TrkB-mediated extracellular signal-regulated kinase 1/2 (ERK 1/2) and AKT signalling to elevate cell proliferation and differentiation into bone.[32] In addition, exosomes released after CNS damage can also promote fracture healing. Studies have shown that CNS neurons damaged after traumatic brain injury release exosomes enriched in miR-328a-3p and miR-150-5p, which target the *FOXO4* and *CBL* genes, respectively, in osteoblastic precursor cells, and high expression of these genes facilitates osteogenesis.[33]

Peripheral nervous system regulation of osteogenesis: Peripheral sensory nerves can release a variety of neurotransmitters as well as neuropeptides to regulate bone metabolism. Norepinephrine released by peripheral sympathetic neurons has several receptor types including α- and β-adrenergic receptor, and each of them contains multiple subtypes, which are expressed by various types of osteoblasts. Norepinephrine may inhibit the proliferation of human BMSCs through β2-adrenergic receptor-induced ERK1/2 phosphorylation and induce osteoclastogenesis through activation of receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin. Furthermore, both α- and β-adrenergic receptor are G-protein-coupled receptors, and the binding of α-adrenergic receptor decreases cyclic adenosine monophosphate levels and subsequently inhibits the PKA pathway and exhibits osteoclastogenic effects, whereas binding to β1-AR produces the opposite effect.[34, 35] The exact mechanism needs to be further verified. In addition, the neuro-factor calcitonin gene-related peptide (CGRP), a major neuropeptide secreted by sensory nerves, promotes BMSC proliferation, recruitment to the site of ossification, differentiation toward osteogenesis, and enhances the expression of alkaline phosphatase and Runx-related transcription factors. Moreover, the receptor for CGRP was highly expressed on differentiated BMSCs, and CGRP promoted osteogenic differentiation of BMSCs through the Wnt/β-catenin protein signalling pathway.[35] Peripheral nerves participate in bone development and repair by secreting neurotransmitters, neuropeptides, and other biologic factor signals. In return, bone can provide mechanical support for nerves. Meanwhile, BMSCs secrete cytokines such as NGF and BDNF during bone regeneration to promote the growth and functional expression of nerve cells, realising the crosstalk between nerves and bone.[36]

Bone lineage cell microenvironment

Functional cells of the bone lineage can be mainly categorised into osteoblasts, osteoclasts, and osteocytes, each of which plays an important role in the different phases of the bone regeneration process (inflammatory, repair, and remodelling phases).

During the inflammatory phase of bone regeneration, osteoclasts are controlled by cytokines and signalling molecules, and the receptor RANK on their surface binds to RANKL secreted by various types of cells in the microenvironment to guide the differentiation of osteoclast precursor cells into osteoblasts.[37] At the same time, osteoclasts bind to the bone surface through receptors such as integrins on the cell surface, forming the basis for adsorption, and enabling them to come into close contact with the bone matrix. Functionally activated

osteoclasts produce acid phosphatase, carbonic anhydrase, matrix metalloproteinases (MMP-9 and MMP-13), and other acids to degrade the bone matrix, achieve bone resorption, degrade damaged bone tissue, and provide space and signals for subsequent repair.[38, 39]

During the repair phase of bone regeneration, osteoblasts become active, promote ossification and remodelling of bone structure, and promote the repair of bone tissue. Growth factors such as TGF-β and BMP in the bone regeneration microenvironment promote the transcription of bone-related genes such as *RUNX2* and osterix, thus promoting osteoblast proliferation and the expression of osteogenic functions.[40] Activated osteoblasts begin to synthesise and secrete collagen and other bone matrix components to support new bone formation. At the same time, osteoblasts resist osteoclastic bone resorption by secreting substances such as alkaline phosphatase and promote bone matrix mineralisation and the formation of calcium salt deposition, which in turn leads to the hardening and maturation of new bone. The bone matrix deposited by osteoblasts gradually forms new mature bone tissue, which fills bone defects or repairs fracture sites.[41]

During the remodelling phase of bone regeneration, osteoblasts are involved in the synthesis of new bone matrix, osteoclasts regulate the removal of damaged bone tissue, and osteocytes maintain bone tissue homeostasis by regulating the bone's metabolic state.

The interactions and signalling pathways between these cells regulate the remodelling and repair process of bone tissue. Osteoblasts regulate their proliferation and differentiation through signalling pathways such as Wnt and BMP that can promote new bone formation and hardening of bone matrix deposition, so that the bone tissue gradually recovers its normal structure.[42] Meanwhile, osteoclasts activate and resorb irregular new bone through the osteoprotegerin/receptor activator of nuclear factor κB (RANK)/RANKL system to balance the resorption and formation of bone.[43] In the later stages of bone regeneration, they participate in regulating the balance of bone resorption and deposition by exchanging substances with surrounding cells and blood vessels, sensing and regulating the metabolic state of bone tissue.

Chemical substance microenvironment

This microenvironment includes bioactive factors and oxygen and is at a specific pH. Bioactive factors are a series of cytokines interacting to form a complex regulatory network that promotes bone tissue regeneration and repair by regulating the proliferation, differentiation, and function of osteoblasts, endothelial cells, immune cells, and neural cells. The pH and aerobic metabolic environment of the bone regeneration microenvironment are also key factors, and the use of pH- or ROS-responsive HMS can regulate the acidity and alkalinity of osteoblasts during apoptosis.[44]

Bioactive factors

In the bone regeneration microenvironment, the bioactive factor environment refers to a series of biomolecules and signalling molecules and their interactions that play a key role in the process of bone tissue regeneration and repair. These bioactive factors interact with each other in a complex manner in the intercellular and extracellular matrix (ECM), regulating and influencing the behaviour and functional expression of osteoblasts and periosteal cells, thereby affecting the process of bone regeneration and repair. In the bone regeneration microenvironment, bioactive factors such as BMP, epidermal growth factor, and fibroblast growth factor[45] can promote osteoblast proliferation and osteogenic differentiation. VEGF can promote the formation of new blood vessels, provide an adequate supply of oxygen and nutrients to bone tissue, and promote bone regeneration.[46] Inflammatory factors such as tumour necrosis factor-α promote the formation and activation of osteoclasts, and the early inflammatory phase regulates bone metabolic homeostasis. In addition, in the process of bone regeneration, bone tissue as well as inflammatory cells can produce NGF. After NGF acts on TrkA receptors of nerve cells, it can promote periosteal neurogenesis and facilitate nerve production of CGRP, which further promotes osteogenesis and achieves neuralised osteogenesis.[47]

In addition, cytokines can interact with each other to form a complex network of signalling pathways, and they regulate osteoblast fate and function. In conclusion, the cytokine milieu in the bone regeneration microenvironment is a complex regulatory network for bone tissue repair and regeneration by promoting processes such as osteoblast proliferation, differentiation, and angiogenesis.

pH and aerobic metabolic environment

Oxygen is an indispensable molecule for maintaining cell viability, growth, metabolism, differentiation, and intercellular communication. The primary cause of tissue hypoxia is disruption of the vascular network at the site of injury, resulting in delayed or undelivered oxygen delivery to the neighbouring cells. Chronic hypoxia often leads to extensive cell death and tissue necrosis, to which highly metabolic and oxygen demanding skeletal cells are more sensitive. Therefore, it is important to ensure an adequate supply of oxygen to hypoxic tissues and to condition cells to adapt to the hypoxic environment. HMS can facilitate *in situ* oxygen production by incorporating oxygen-producing components into biomaterials. For example, Guan et al.[48] prepared HMS with a shell-core structure by combining hydrogen peroxide with the outer shell of microspheres, which would provide oxygen to the wound environment and facilitate tissue regeneration when the outer layer of hydrogen peroxide binds to hydrogen peroxidase.

In bone tissue, the pH in the bone microenvironment is preserved mainly by the secretion of acidic or alkaline substances by osteoblasts during bone metabolism. The appropriate pH for bone regeneration usually ranges from neutral to slightly alkaline. Fliefel et al.[49] concluded that pH 8.0 and 8.5 are appropriate for promoting the proliferation and osteogenesis of human BMSCs toward differentiation. Different acidic and alkaline microenvironments have different effects on bone regeneration under different pathological conditions. Within the normal pH range, it can maintain the activity and function of osteoblasts, promote the formation and deposition of the bone matrix, maintain normal cell-cell interactions, inhibit bacterial

growth, and promote the generation of new blood vessels and blood supply. During bone regeneration, early apoptosis is often accompanied by changes in energy metabolic pathways, such as glycolysis and the tricarboxylic acid cycle. These changes may lead to the production of large amounts of lactic acid and other metabolites within the cell, causing acidification of the cell's surroundings. In the late stages of apoptosis, cells may be lysed and release factors such as free fatty acids and ROS, which can affect the pH of the surrounding environment and increase the ROS levels. Therefore, some researchers have utilised these microenvironmental properties to develop HMS with pH or ROS responsiveness, and these microspheres are used in bone regeneration therapy.[50] These microspheres are designed to precisely release drugs, growth factors, or stem cells at the site of bone injury or defect to promote bone tissue regeneration and repair. The uniqueness of this technology lies in its sensitive response to the pH or ROS of the surrounding environment, allowing it to adjust the release rate and pattern in response to changes in the tissue microenvironment.

Physical microenvironment

In bone regeneration, the physical microenvironment includes mechanical, photothermal, electromagnetic, and ultrasound forces. These physical stimuli can promote osteoblast proliferation, migration, and osteogenic differentiation, which in turn promote bone tissue regeneration. Mechanical force signals and magnetic force are involved in regulating osteoblast behaviour and inducing osteogenic differentiation by altering the calcium ion level in osteoblasts, thus promoting the process of bone regeneration. Photothermal stimulation and electrical stimulation can increase the metabolic activity of cells and mobilise cell proliferation and migration; in addition, ultrasound, as a form of mechanical energy, can be transmitted into the body to produce unique biological effects, and low-frequency ultrasound has a positive effect on the proliferation, activation, and mineralisation of osteoblasts. The positive effects of low-frequency ultrasound on osteoblast proliferation, activation, and mineralisation have been described by Kang et al.[51]

Bone is a mechanosensitive tissue that responds to mechanical signals from its environment through mechanotransduction. Mechanical stimuli significantly influence the development and remodelling of skeletal structures. In bone tissue engineering, bioscaffolds are often required as temporary structural supports to fill bone defects, withstand early mechanical loads, and transmit external mechanical forces to mobilise osteoblasts and guide new bone formation.[52] Furthermore, it has been shown that mechanical tension induces antioxidant effects through activation of the adenosine monophosphate-activated protein kinase-silent information regulator type 1 signalling pathway and calcium channels in osteoblasts to restore osteoblast-associated osteogenic gene expression.[53] Similarly, an extremely low frequency magnetic field (50 Hz, 0.8 mT) can promote bone regeneration by modulating calcium channel activity in osteoblasts to induce calcium uptake by osteoblasts.[54] In addition, light and thermal responses as external stimuli can significantly promote bone regeneration. Types of light can include ultraviolet, visible light, near-infrared (NIR), and lasers of different wavelengths. Among them, NIR light is considered effective in promoting bone regeneration by enhancing cellular metabolic activity and modulating cellular pathways.[55] A study has shown that low intensity NIR induces a reduction in the expression of the biological clock protein cryptochrome 1 (*CRY1*), which activates the BMP signalling pathway and promotes the expression of the *RUNX2* and osterix genes to promote bone regeneration.[56] The synergistic effect of light and thermal stimulation has been shown to be effective in promoting bone regeneration. The photothermal effect produced by NIR light can modulate photothermal biomaterials, control their appropriate temperature, and induce the expression of heat shock protein 70, alkaline phosphatase, and other osteogenesis-related proteins in BMSCs, which promotes the differentiation of BMSCs into osteoblasts.[57]

The application of electrical stimulation in bone tissue engineering offers a promising approach to promote bone regeneration. Bone inherently exhibits piezoelectric properties, generating electrical and biochemical signals in response to the mechanical activities of bone remodelling and repair. Through electrobiological effects such as charge separation and potential changes when piezoelectric materials are subjected to mechanical strains, localised electric fields are formed, which promote bone tissue regeneration and repair through the activation of calcium channels as well as intracellular calmodulin signalling pathway, thereby inducing osteogenic gene expression in osteoblasts and promoting bone tissue regeneration and repair.[58] In addition, ultrasound-responsive biomaterials can directly or indirectly transmit signalling molecules with the help of ultrasound stimulation, which is often used in bone regeneration therapy.[59] Moreover, low-intensity pulsed ultrasound (LIPUS) is the most widely studied technique in the field of ultrasound stimulation for bone repair, and its biological response is complex, involving multiple cell types and pathways. Miyasaka et al.[60] found that LIPUS activates the BMP signalling pathway and the pathway downstream of SMAD1, which induces osteoblast activation and osteogenic signalling. In addition, LIPUS induces the release of adenosine triphosphate and cell differentiation via the P2X purinoceptor 7 receptor on the membrane of osteoblasts, and adenosine triphosphate, as a key mediator in the mechanical stimulation response, further induces bone formation.[61] Although clinical and experimental studies have demonstrated that LIPUS has an enhancing effect on bone regeneration, the physiological mechanisms involved in the complex bone healing process remain unclear and require further investigation.

Introduction to Hydrogel Microspheres

Materials for hydrogel microsphere preparation

HMS materials can be categorised into natural polymer HMS materials and synthetic HMS materials. Natural polymer hydrogels typically rely on physical cross-linking or natural cross-linking agents, while synthetic hydrogels generally involve chemical cross-linking reactions. Furthermore, natural polymer hydrogels are frequently employed in biomedical applications given their exceptional biocompatibility. In contrast, synthetic hydrogels have a diverse range of applications, including environmental monitoring and smart

materials. Different HMS materials play important roles in the bone regeneration process, and their properties and functions are crucial for regulating the growth, repair, and regeneration of bone tissue. Selecting appropriate HMS materials and precisely regulating them according to their properties promotes the proliferation and differentiation of osteoblasts and the formation of new bone tissue, thus accelerating the bone regeneration process.

Natural polymer hydrogel microsphere materials

Natural polymers are produced by photosynthesis or biochemical reactions in nature or they can be derived from natural products. Owing to their diverse properties of biocompatibility, biodegradability, and environmental friendliness, these polymers serve as ideal backbones for the fabrication of hydrogels. Natural polymer HMS materials consist of nanoscale microspheres derived from natural polymer hydrogels. Natural polymer HMS materials are materials in the form of nanoscale microspheres based on natural polymers.[62] They are usually constructed from natural macromolecules such as proteins and collagen and therefore have good biocompatibility. These natural polymers can be gradually broken down and metabolised in living organisms, better adapting to the biological environment, reducing adverse reactions, and promoting bioabsorption of materials. Different natural polymer materials have different biofunctional properties; below, we have reviewed several common natural polymer HMS materials:

Collagen microspheres: These exhibit biocompatibility, as collagen is a natural component of human tissue and is less likely to provoke an immune response. Furthermore, collagen hydrogels have garnered significant attention with respect to their capacity to replicate the natural microenvironment of articular cartilage, showcasing outstanding biocompatibility and bioactivity.[63] However, collagen microspheres still have the disadvantages of poor short-term effects and high cost.[64]

Compared with the poor short-term effect of collagen microspheres, the release rate and duration of silk fibroin microspheres can be controlled by adjusting the structural and chemical properties of the microspheres, thereby realising the precise regulation of the drug or active ingredient release. In addition, the silk fibroin microspheres also have good biocompatibility and degradability and can be used in various fields such as tissue engineering and biosensing and have a wide range of application prospects. Wang et al.[65] utilised the low cytotoxicity and non-immunogenicity of regenerated silk filament protein nanodrug carriers to achieve effective encapsulation of regenerated silk filaments and controlled drug release. This approach enhanced the stability of bioactive molecules within OA lesions, thereby improving the therapeutic effect. Moreover, the preparation process of silk protein microspheres is relatively complex and includes extraction from the silk protein, microsphere preparation, and surface modification, all of which require a level of technical and equipment support.[66, 67]

Fibrin microspheres: Fibrin's precursors, fibrinogen, and thrombin (which can be extracted from the patient's own blood) can create fully autologous scaffolds that provide some degree of protection to the drug, thereby prolonging its presence in the body. Additionally, the combination of fibronectin with other nanomaterials can more effectively mimic the natural nanostructural features of bone, thereby promoting bone regeneration. In addition, fibrin microspheres have a high drug loading capacity that can effectively load the drug inside the microspheres and improve its bioavailability. However, fibrin microspheres also have some limitations, such as poor water solubility, which may lead to increased processing difficulty during preparation and affect the stability and quality of the microspheres. Moreover, given the different sources and extraction methods of fibrin, there may be some differences in its purity that could affect the biocompatibility of microspheres and subsequent drug release.[68, 69]

Gelatine microspheres (GMs) are considered to be one of the most widely used materials for the preparation of HMS. Hayashi et al.[70] demonstrated that gelatine HMS improved the viability and osteogenic differentiation of MSCs by observing the proliferation and osteogenesis-related gene expression of MSCs in GMs co-culture with rat BMSCs. Gelatine has a fibrous structure similar to that of collagen, which helps to mimic the structural characteristics of natural bone tissue, providing a suitable three-dimensional (3D) scaffolding and growth environment that contributes to osteoblast attachment and growth.[71] Meanwhile, gelatine usually contains Arg-Gly-Asp peptide (RGD) sequences on its surface, which is a bioactive peptide sequence that can bind to cell surface integrin receptors that is conducive to promoting osteoblast adhesion and proliferation.[72] However, GMs still have the disadvantages of inadequate mechanical properties and excessive degradation rate.

Chitosan HMS: Chitosan hydrogels usually have better mechanical properties with respect to strength and stability to provide better support and mechanical stimulation for the bone regeneration environment. Furthermore, chitosan HMS has antimicrobial, anti-inflammatory, and healing-promoting bioactivities; for example, Gao et al.[73] have demonstrated the ability of chitosan HMS to improve bone regeneration and healing through the design of silver nanoparticle-loaded chitosan/hyaluronic acid porous microspheres for antimicrobial use to alleviate the localised malignant inflammatory environment brought about by infected wounds. The controlled release of chitosan HMS can also be achieved by changing the degree of cross-linking of chitosan hydrogels.[74] However, the ability of chitosan microspheres in functionalisation is more limited, making it difficult to achieve complex functionalisation modifications, such as targeting delivery or cell recognition properties.

Chondroitin sulphate microspheres: Chondroitin sulphate is a crucial component of ECM in medullary tissues, offering several advantages including a diverse range of sources, excellent biocompatibility, high stability during transportation, and cost-effectiveness.[75-77] In this context, Hong et al.[76] designed chondroitin sulphate-functionalised microspheres loaded with

IL-1 receptor antagonist. This HMS demonstrated the ability to reduce the inflammatory response of the intervertebral disc, maintain disc height and nucleus pulposus water content, and preserve the structural integrity of the disc, among other beneficial effects. Moreover, the size, shape, and drug-release characteristics of chondroitin sulphate microspheres can be controlled by adjusting the preparation process and formulation to meet different clinical needs. However, compared to other microspheres, chondroitin sulphate microspheres have the disadvantages of a complicated preparation process and poor stability.[77]

Synthetic HMS materials

Synthetic hydrogels are materials composed of synthetic polymer networks. Moreover, synthetic HMS materials have better controllability, tunability, stability, and mass production than natural polymer HMS materials, making them more suitable for some specific application scenarios. However, synthetic polymer hydrogels exhibit lower biological activity than natural hydrogels.[78] The selection of the specific type of material and method ultimately depends on the requirements and nature of the intended application.

Poly(lactic-co-glycolic acid) (PLGA) microspheres: PLGA microspheres are highly versatile. Targeting ligands (e.g. RGD peptide) for receptors on the surface of bone tissue cells are attached to the surface of the PLGA microspheres to improve their recognition and target osteoblasts for more accurate osteogenic delivery. By using PLGA microspheres to load osteogenic factors (such as BMP-2),[79] the adhesion between microspheres and bone tissue cells is enhanced, while promoting the functional expression of bone cells, achieving more efficient HMS delivery of drugs to promote bone regeneration.

Gelatine methacrylate (GelMA) microspheres: GelMA microspheres have stronger biocompatibility and a more mature preparation process than PLGA microspheres. PLGA microspheres release metabolites such as lactic acid and hydroxypropionic acid during degradation, which may lead to a change in the local pH level, affecting the physiological environment of the surrounding tissues.[80] GelMA is synthesised through the chemical modification of gelatine with methacrylic anhydride. The RGD sequence present in gelatine enhances biological interactions between cells and scaffolds. Moreover, modified gelatine offers cell adhesion sites and MMP hydrolysis sites, which promote cell proliferation and distribution. The incorporation of methacrylic acid groups in GelMA imparts photosensitivity, enabling cross-linking through exposure to ultraviolet light, thereby forming hydrogels. This photo-crosslinked hydrogel is biocompatible, exhibits controlled biodegradability, and has low immunogenicity, presenting unique advantages for tissue regeneration in applications such as bone, cartilage, myocardium, and blood vessels.[81] Consequently, GelMA hydrogels are extensively utilised in bone regeneration engineering.

Platelet-derived growth factor (PDGF) mimetic peptide microspheres: PDGF mimetic peptide microspheres are anticipated to enhance bone regeneration because of their capacity to replicate the effects of PDGF, serving as effective pro-dividing agents and chemoattractants for stem cells. During the initial stages of bone injury, PDGF is released by platelets and macrophages, which not only stimulates local angiogenesis but also orchestrates the osteogenic pathway, thereby facilitating accelerated bone regeneration.[82, 83] In addition, PDGF mimetic peptide microspheres also have the advantages of controlled release and slow drug release.[84]

In addition to the above HMS materials, several other HMS materials are also summarised below along with their advantages and disadvantages (**Table 1**).[62, 64-66, 71-75, 85-92]

Preparation of hydrogel microsphere

Batch emulsification technology

Typically, a hydrogel precursor solution and immiscible oil are used as the hydrophilic and hydrophobic phases, respectively, to create droplets. The hydrogel prepolymer is added dropwise to the oil containing the cross-linking agent and surfactant, and mechanical agitation is performed to homogenise the solution to form aqueous droplets suspended in oil. The duration and degree of agitation affects the size and dispersion of the droplets, and the remaining oil in the droplets is then removed by repeating the centrifugation, washing, and filtration steps.[12] Photo cross-linking is a commonly used method to cross-link droplets suspended in the oil phase by an external light source; in addition, temperature changes (e.g. heating or cooling) can be used to induce cross-linking of thermosensitive hydrogels, and these methods are compatible with cell encapsulation.[93, 94] Although the intermittent emulsion method is simple, the fact that the emulsification procedure varies each time leads to increased variation between batches, making the resulting hydrogels more polydisperse. Polydispersity has a significant impact on many applications of hydrogels, e.g. it is challenging to control the number of cells in each droplet in hydrogels containing multiple droplets.

Microfluidic emulsification technology

Droplet-based microfluidics is a typical method for achieving fine control. Controlled droplets can be obtained by regulating the flow rate of aqueous solution and oil at the intersection point. In the water-in-oil system, shear forces and hydrophobic interactions at the intersection point contribute to droplet formation. Adjustment of the relative flow rates of the two phases and the intersection geometry (e.g. diameter, shape, and structure) can be used to regulate the range of micro-hydrogel diameters (5–500 μm).[95, 96] Microfluidic technology is also suitable for the encapsulation of various biologics, whereas monodisperse HMS provides better control over the drug release profile. For cell encapsulation, the number of cells within a single micro hydrogel can be counted by doping a certain number of cells into the hydrogel precursor solution.[97-99] Microfluidic-based deformational cytometry can also be realised by this technique, which can be used to study cell biomechanical properties and clinical diagnostic applications owing to its good accuracy.[100] In addition, monodisperse micro-hydrogels with controllable diameters can be used as high-performance bio-lubricants to reduce friction on sliding surfaces.[101]

Table 1. Hydrogel microsphere preparation materials and their advantages and disadvantages

Raw material	Preparation method	Advantage	Disadvantage
Natural polymer hydrogel microsphere			
Collagen[62]	Emulsification	1. Cell adhesion and growth; 2. Bioactivity	1. low mechanical strength; 2. Immunogenicity risk; 3. Rapid degradation rate
Gelatin[71, 72]	Emulsification; Microfluidics; Electrospray	1.Good biocompatibility; 2. Easy processing and low cost; 3. Functionalization and cross-linking	1. Low mechanical strength; 2. Rapid degradation rate and degradation byproducts
Chitosan[74]	Emulsification; Electrospray	1. Safe degradation byproducts; 2. Antimicrobial properties	1. Allergy risk; 2. Low cellular interactions; 3. Low degradation rate
Silk fibroin[64-66]	Microfluidic; Electrospray	1. Cell Adhesion; 2. Good mechanical strength	1. Hydrophobicity; 2. Slower degradation rate
Chondroitin sulfate[75]	Emulsified	1. Anti-inflammatory, antioxidant, regulates enzyme activity, cellular activity, and communication; 2. Negative electronegativity	1. Rapid degradation; 2. Limited mechanical strength
Fibrous protein[64-66]	Emulsified; Electrospray	1. Mimics natural extracellular matrixc; 2. Facilitates vascular regeneration and cytokine adhesion	1. The preparation process is complex and costly; 2. Potential for infection
Chitin[85]	Emulsified	1. Good biocompatibility; 2. good biosorption properties	1. High production costs; 2. Lower mechanical properties
Synthetic hydrogel microsphere			
Alginate[86]	Emulsified; Microfluidic; Electrospray	1. Good biocompatibility; 2. Easy to cross-link; 3. Ease of Gelation	1. Low degradation rate; 2. Sensitivity to pH
Polylactic acid-hydroxybutyric acid copolymer[71]	Emulsification; Electrospray	1. High tunability; 2. Acceptable mechanical strength	1. Structural Instability; 2. Acid product
Gelatin methacrylate[87]	Emulsification; Microfluidics; Electrospray	1. Adjustable mechanical properties; 2. Adjustable physicochemical properties	1. Limited mechanical behavior; 2. Complex preparation
Polycaprolactone[88]	Microfluidic; Electrospray	1. Acceptable biocompatibility; 2. High mechanical strength	1. Acidic products; 2. Slow degradation rate
Cellulose compound[89]	Emulsified	1. Good biocompatibility; 2. High mechanical strength and stable chemical structure	Poor flexibility and water solubility
Polyethylene glycol[90]	Emulsified; Microfluidics	1.High hydrophilicity, low immunogenicity, highly biocompatible, biodegradable; 2. Easy to modify and functionalize	1. Poor biological activity; 2. Limited mechanical strength
Polyvinyl alcohol[91]	Emulsified; Microfluidics; Electrospray	1. High water solubility, high biocompatibility; 2. Easy to modify and functionalize.	1. Limited biological activity; 2. Limited mechanical properties
Poly(lactic-ethanolic acid)[92]	Emulsified; Electrospray	1. Adjustable physical properties; 2. High hydrophilicity	Less hydrophilic and biologically active.
Platelet-derived growth factor mimetic peptide[73]	Electrospray	1. High biological activity; 2. Low immunogenicity; 3. Stronger stability	1. Higher production costs; 2. Limited biosafety; 3. Low mechanical strength

The main advantage of microfluidics is its good control of droplet generation, which allows the size and structure of micro-hydrogels to be effectively regulated by adjusting the input flow rate and channel geometry. The main limitations of microfluidic methods compared to intermittent emulsion methods are the relatively low throughput and the need for advanced and expensive equipment. However, it is much more difficult to produce smaller diameter micro-hydrogels, because the production volume is negatively correlated with the micro-hydrogel diameter. To address this problem, advanced microfluidic devices with multiple connectors have been developed, hence making it possible to produce multiple micro-hydrogels in a single device.[99]

Lithography

In imprint lithography, we load a hydrogel precursor into a templated mould with negative features of the desired HMP for cross-linking and curing, and the lithography process uses a templated photomask that selectively cures specific regions of the hydrogel precursor to form the HMP.[10] The main advantage of lithography is its ability to tightly control the geometrical features of the mask or mould, which allows for

significant control over the HMP geometry. With advances in microfabrication technology, we can now produce moulds and masks with nanoscale features that make it possible to fabricate designer HMPs with customised internal and external architectures and monodispersity.[12, 102, 103]

However, the imprint-photolithography approach has several shortcomings. One of these is the need to remove the cross-linked HMP from the mould, which limits the complexity of the achievable internal or external complex features. Photolithography can only produce relatively simple geometries with relatively low yields.[12, 104] Specifically, particle production rates are limited by the size of the mould or mask that can be prepared with the available microfabrication techniques and the field of view of the light source or objective.

Electrospray

Spraying is the simplest method to prepare HMS for industrial production. The principle is that the prepared solution or emulsion is dispersed into very small particles by means of an atomiser, which separates the particles from water vapor and collects the particles to obtain HMS.[105] The electrospraying provides more precise microsphere size control than the spray method. During electrospraying, the monomer solution is pumped through a syringe and an electric field is applied at the end of the needle. When the voltage exceeds a critical threshold for overcoming surface tension, the monomer solution forms charged droplets that are attracted to a designated collector.[106] These charged droplets solidify in the collector and can measure as small as 1–2 μm depending on conditions such as polymer flow rate, applied voltage, and needle diameter.[107] The main advantages of the electrospray methods are their speed and simplicity, and their ability to rapidly generate large numbers of particles in a single process. However, a major limitation is the lesser control over the individual particles formed, leading to the generation of polydisperse distributions (**Figure 2**).[108]

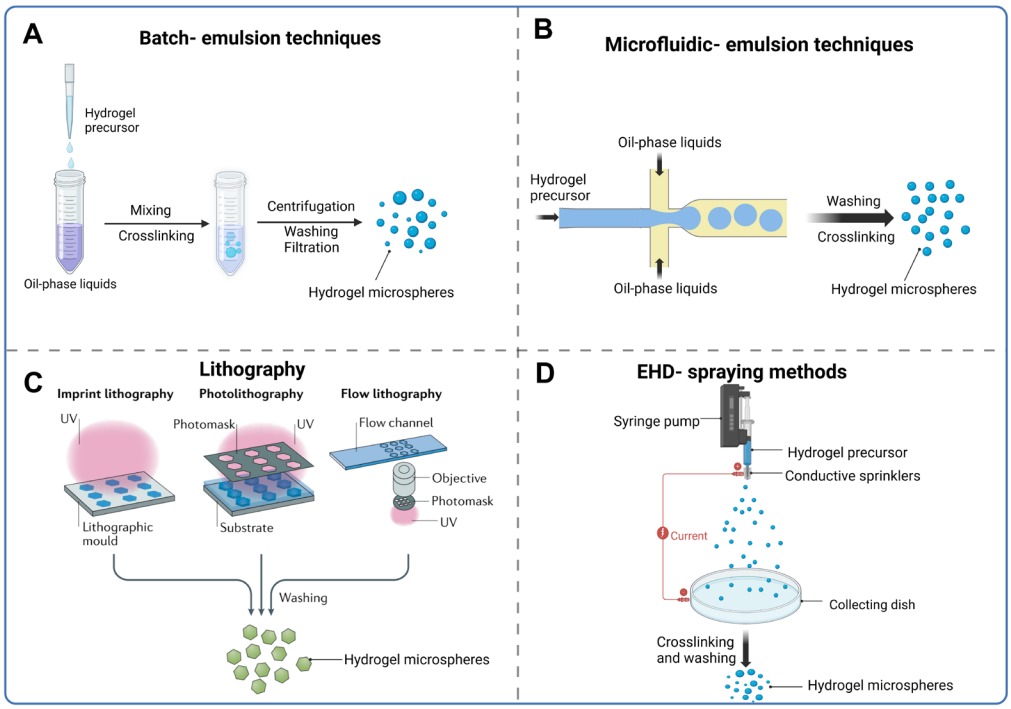

Figure 2. Examples of HMS preparation techniques. (A) Batch emulsification uses immiscible liquids (e.g. water and oil) mixed together to produce hydrogel particles (HMS) that can be cross-linked to form a hydrogel. (B) Microfluidic emulsification uses a flow of immiscible liquids to create droplets that can be cross-linked to form an HMS. (C) Photolithography utilises photomasks or moulds that are used as templates for the micro-hydrogel. (D) Electrospray method uses electricity to cause the flowing solution to form charged to form droplets which can then be cross-linked to form an HMS. Created with BioRender.com. EHD: electrohydrodynamic; HMS: hydrogel microsphere; UV: ultraviolet.

Functional characteristics of hydrogel microspheres

HMS is characterised by adjustable porosity and space, controlled release, scaffold function, and multifunctional carrier. Its good pore structure facilitates cell attachment, proliferation, differentiation, and the diffusion and release of bioactive substances. Changing the chemical composition, pore structure, and surface modification can achieve sustained and controlled drug release, optimise therapeutic effects, and reduce side effects. It can be used as a scaffold for cell culture, tissue engineering, and regenerative medicine. It has good biocompatibility and hydration, provides a good cell adhesion surface, and promotes cell proliferation and differentiation while also providing a temporary support structure. It can also carry a variety of drugs or bioactive substances to achieve multi-effect therapy, and it can be modified or functionalised by surface modification to achieve targeted delivery and

therapy. Furthermore, it can be used in combination with other materials to construct composite systems, further expanding its application potential in the biomedical field.

Pore space and porosity

The interstitial spaces between the HMS are referred to as the pores of the granular scaffold, and the size of the pores is proportional to the size of the HMS. Thus, filled HMS form micron-sized pockets of interstitial space, and cells with diameters on that length scale can easily penetrate and pass through the granular scaffolds without the need to degrade the hydrogel. HMS has higher porosity, more uniform pore size distribution, adjustable pore size, and larger specific surface area than bulk hydrogels; the microporosity of HMS increases fluid flow, mass transfer, permeability, and cell permeability, which facilitates cell adhesion, proliferation, and maintenance of cellular differentiation phenotype.[12, 109]

In addition, HMS can be prepared as a porous structure, and the pore structure can be designed independently according to the size of the carrier cells or the size of the drug molecules, which facilitates cell penetration and reaches the target site or aids in slow release of the drug.[110] The high specific surface area and high porosity of HMS can facilitate cell-cell interactions, nutrient exchange, and metabolic waste discharge, as well as functionalised modification and efficient delivery of drugs and bioactive factors.[111]

Adjustable release

The pore structure of HMS can be designed by modulating the preparation conditions and material selection, thus affecting the diffusion rate and release rate of the drug or the active ingredient in it. Adjusting the degree of cross-linking of HMS can affect its stability and drug release behaviour, enabling control of the release rate of active ingredients.[112] Using responsive pH-sensitive and temperature-sensitive hydrogel materials, controlled release of active ingredients can be ensured in response to changes in external conditions.[113] In addition, HMS can be designed as a composite scaffold with a shell-and-core structure and HMS for sequential release. HMS with shell-and-core structure have two parts: the core part usually contains one or more active substances, and the shell part contains different polymers to control the release rate of the active substances and protect the core content from premature release. This structure allows the microspheres to gradually release the outer drug layer first through the shell, followed by a gradual release of the drug in the core according to the degradation or permeation properties of the shell, resulting in a sequential, controlled release.[114] In HMS composite scaffolds, HMS are usually integrated into biocompatible materials. This composite structure not only protects the microspheres from mechanical stresses but also allows for further modulation of drug release through the design of the scaffolds by adjusting the pore size of the scaffolds or the rate of biodegradation or via its hydrophilic and hydrophobic properties.[115]

Three-dimensional platform construction (scaffolding)

Microspheres can be delivered as is or used as building blocks for macroscopic scaffolds to further localise the microspheres to the desired site. These scaffolds are commonly implanted in bone defects as temporary space fillers designed to provide initial mechanical support during the early stages of bone healing. Two main types of microsphere-based constructs of bone tissue engineering scaffolds are currently available—microsphere-incorporated scaffolds and microsphere-based scaffolds.

Microsphere-incorporated scaffolds: These refer to the construction of scaffolds that use microspheres as discrete components embedded in a continuous matrix. The continuous matrix can be a polymer or a ceramic. The mechanical properties of composite scaffolds are usually determined by the continuous matrix. However, the size and density of the microspheres also affect the mechanical properties of the scaffold when the microsphere concentration is significant.[12] An advantage of microsphere-incorporated scaffolds is that the properties of the microspheres and the continuous matrix can be adjusted simultaneously to optimise the release pattern of bioactive factors.

Microsphere-based scaffolds: Following a bottom-up construction strategy, microspheres can be used as building blocks for scaffold fabrication, resulting in microsphere-based scaffolds. In this scaffold type, the interstitial space present between microspheres is sufficient to allow oxygen and nutrients to enter while supporting inward cell growth.[116] The three main packaging strategies for microsphere-based scaffolds are randomised packaging, self-assembly, and 3D printing, each of which has its own applicable scenarios and features, and the selection of the appropriate strategy depends on the specific application needs and requirements. In randomised packaging, microspheres are arranged or mixed together in an unorganised manner to form the structure of the scaffold. This packaging strategy is simple and easy to implement and is suitable for some simple scaffold structures. The advantage is that the preparation process is simple, but the disadvantage is that the structure is not sufficiently organised, which may affect the performance and stability of the scaffold. Self-assembled packaging utilises the interaction force between the microspheres, wherein the microspheres are spontaneously assembled into an ordered structure under certain conditions. This packaging strategy produces scaffold structures with better ordering and control and is suitable for applications that require precise control of structural morphology. The advantage is that highly ordered structures can be achieved, but precise control of the preparation conditions is required. 3D printing technology directly stacks and cures microsphere materials layer-by-layer according to a designed model to form a scaffold with a predetermined structure. A highly customised scaffold design can be achieved and is suitable for the preparation of various complex structures. The advantage is that highly customised and complex structures can be prepared, but the cost of equipment and materials is relatively high.[90]

Multifunctional carriers

HMS can efficiently carry various types of drugs including chemical drugs, protein drugs, and nucleic acid drugs. Owing to the large specific surface area and high porosity of HMS, these drugs can be encapsulated inside the microspheres or adsorbed on the surface of the microspheres for protection as well as high loading rates for localised or systemic therapies

in tissue engineering.[117] HMS as a carrier can improve drug stability, prolong the duration of efficacy, and reduce toxic side effects.[118] Therefore, HMS can carry bioactive molecules such as growth factors and cytokines, which can be used to promote tissue regeneration and repair, such as promoting angiogenesis and bone tissue regeneration. At the same time, HMS can be used as the cellular carrier for various types of cells such as stem cells and fibroblasts, for cell transplantation therapy in tissue engineering and regenerative medicine. In addition, HMS can carry fluorescent markers for cell tracking, biomolecule detection, and other applications. By integrating biosensing elements into HMS, biomolecules can be detected and monitored for use in biosensors and diagnostic devices, which can help in the early detection of diseases and monitoring of biomarkers.[119]

Use of Hydrogel Microspheres in Bone Tissue Engineering

In this section, we present recent research advancements in the utilization of HMS systems as drug and cell carriers, as well as bioscaffolds, to enhance efficient bone regeneration engineering. Furthermore, we outline the applications of the HMS system in the treatment of bone tissue engineering and conditions related to sports medicine in **Figure 3**.

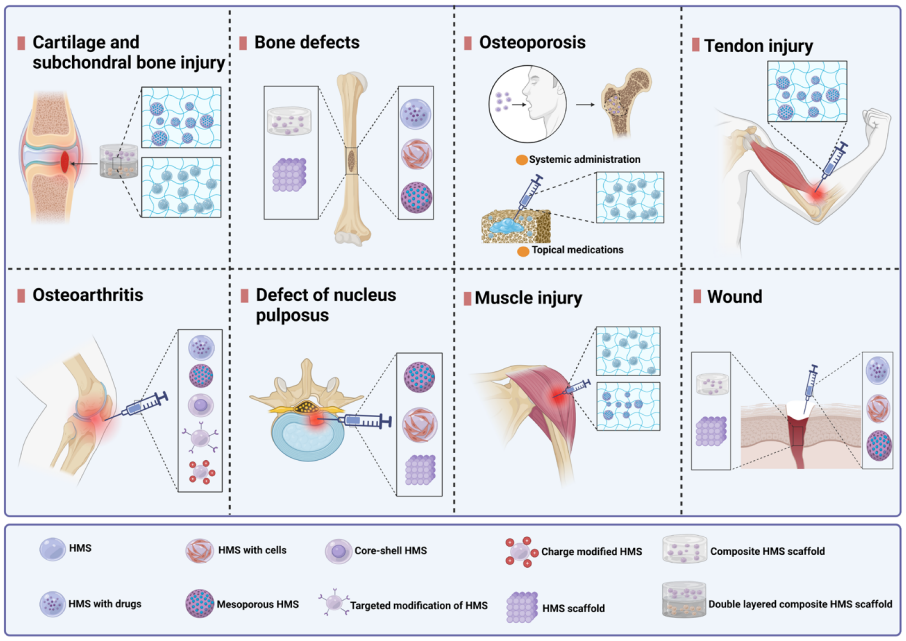

Figure 3. Schematic diagram of HMS for the treatment of locomotor system diseases. Created with BioRender.com. HMS: hydrogel microspheres.

Hydrogel microsphere pharmaceutical compartment engineering

HMS has a promising future as a drug carrier for efficient loading, effective protection, and controlled release of drugs and bioactive factors. This overcomes many limitations of traditional drug delivery methods, such as oral and intraventricular administration which usually require high dosage and repetitive administration and may lead to off-target effects. Currently, bioactive factors are loaded into microspheres by using covalent immobilisation, physical adsorption or precursor solution mixing, and embedding, and the release of the drug is regulated by various HMS modifications.[120]

Hydrogel microspheres for controlled drug release

The release time of drugs can be regulated by adjusting the physicochemical properties of microspheres or modifying microspheres. For example, He et al.[121] developed liposomal chitosan methacrylate HMS loaded with glycyrrhizin (liquiritin), which achieved a dual blocking effect of liquiritin release because of the network of lipid membranes and hydrogel matrices and exhibited significant slow-release kinetics (**Figure 4A**). Other studies prepared sustained-release microspheres for continuous and long-lasting cartilage permeation of drugs by compositing a cationic nanocarrier namely polyamidoamine, with methacrylated hyaluronic acid using microfluidics. The positively charged polylysine was uniformly modified on the surface of the negatively charged methacrylated hyaluronic acid microspheres through charge dipole interaction. The positively charged polylysine on the surface enabled the microspheres to target adsorption on the cartilage surface under the attraction of negatively charged proteoglycans in the cartilage, realising the targeted drug release to alleviate OA (**Figure 4B**).[122] In contrast to the slow drug release feature of HMS in bone tissue engineering, HMS can also precisely and rapidly release drugs for the treatment of OA. Xiang et al.[123] prepared a MMP13-responsive HMS system through the degradation of MMP13 substrate peptide upon binding to MMP13 present in OA. This allows the HMS system to

precisely and rapidly release anti-inflammatory drugs for OA treatment, effectively slowing down disease progression and promoting articular cartilage repair (**Figure 4C**). To achieve the delivery of multiple bioactive factors with programmed release kinetics, microspheres carrying various factors were used to promote bone regeneration. Microspheres carry a variety of drugs to promote bone regeneration and can also enable angiogenesis and modulate inflammation. An increasing number of microspheres are being developed for the delivery of multiple factors. The effectiveness of bone regeneration is highly dependent on the duration of inflammation. Therefore, controlling the duration of early inflammation by precisely modulating biomaterials to promote the interaction between BMSCs and macrophages contributes to short-term inflammation and is more compatible with physiologic bone healing.[124] Accordingly, researchers fabricated a porcine small intestinal submucosa hydrogel containing LL37 peptide and PLGA microspheres encapsulating the WP9QY peptide. The LL37 peptide, which is first released, has good antimicrobial properties and BMSC recruitment capacity, thereby preventing infections at early stages, increasing the number of BMSCs in the damaged area, and promoting early macrophage M1 phenotype. Subsequently, the released WP9QY peptide in microspheres induced more macrophages to convert to the M2 phenotype and promoted the osteogenic differentiation of BMSCs; thus, the sequential release of HMS multi factors for bone regeneration was achieved (**Figure 4D**).[125] In addition, BMSC-exosomes could recruit stem cells for bone repair. Neovascularisation is the main channel for stem cells to migrate to the site of injury, and these exosomes cannot be transported until the angiogenic stage. Most exosomes are phagocytosed by macrophages during the acute inflammatory stage.[126] To address these issues, Huang et al. designed an injectable MMP1-sensitive HMS (KGE) made using a microfluidic chip prepared by mixing self-assembling peptide (KLDL-MMP1), GelMA, and BMSC-exosomes. In the presence of 0.7 ng/mL MMP1, KGE sustained slow release of exosomes for more than 15 days *in vitro*. For more than 4 weeks *in vivo*, the KGE microspheres exerted an enzyme-sensitive release effect and could serve as an ideal delivery material for exosomes.[127] Different bioactive factors can be separately encapsulated into different chambers of the multi-compartment microspheres, ensuring that the factors are individually encapsulated and do not interact with each other, which can be used to achieve synergistic therapeutic effects. Core-shell microspheres are typical multicompartmental shaped microspheres with a shell layer that protects the drug inside the core and reduces the abrupt release of the drug to some extent. Li et al.[128] developed a pH-responsive shell-core structured micro/nano HMS loaded with polyhedral oligomeric sesquicarbazide by using gas microfluidics and ionic cross-linking. The HMS core encapsulated PDAP (polyhedral oligomeric silsesquioxane/desferrioxamine@aspartic acid 8/polyethylene glycol) nanoparticles in the HMS cores, and these microspheres were cross-linked with calcium ions used for the prevention and treatment of osteoporosis. Chitosan and calcium alginate (ALG) microspheres were bound to the inorganic hybrid nanoparticles surface by electrostatic interactions to form a complete shell/core structure. HMS achieved oral drug delivery, gastric protection, enteral slow release, and controlled release, and actively targeted the bone tissue (**Figure 4E**).

Hydrogel microsphere piggybacking on drugs

Application of molecular drugs: In the bone regeneration setting, HMS can promote the improvement of the bone regeneration microenvironment by delivering drugs. Some common molecular drugs such as metal ions, bioactive proteins, and hormones can be applied to bone regeneration therapy by injection or local administration. It has been shown that magnesium ions (Mg^{2+}) bind to bone repair materials and provide composites the ability to promote vascular repair and enhance osteoblast adhesion. Therefore, Zhao et al.[129] prepared a GelMA microsphere using a microfluidic device and grafted a bisphosphonate on its surface. Then, they utilised the coordination reaction of bisphosphonate with Mg^{2+} to achieve the microspheres with strong Mg^{2+}-capture ability and slow-release properties, which afforded the promotion of osteogenesis and angiogenesis by stimulating osteoblasts and endothelial cells and inhibiting osteoclasts, ultimately effectively promoting the regeneration of cancellous bone (**Figure 5A**). The earliest and most widespread application of microspheres in bone defects is their use as delivery carriers for bioactive factors like BMP-2. Jiang et al.[130] found that Mg^{2+} could significantly increase cellular bioenergetic levels through the Akt-glycolysis-mitochondrial RNA splicing 2-mitochondrial axis in support of osteogenesis and consequently, enhance the osteoinductive properties of BMP-2. Thus, the efficacy of low-dose BMP-2 was enhanced in the presence of Mg^{2+}. Methacrylated ALG microgel scaffolds containing Mg^{2+} and BMP-2 were utilised for bone regeneration by preparing the scaffolds (**Figure 5B**). In addition to the above drugs, Li et al.[131] verified the potential of injectable HMS piggybacked with simvastatin for bone repair and regeneration by injecting HMS encapsulated with simvastatin into the post-extraction socket and observing the generation of new bone in the socket. Xu et al.[132] synthesised hollow porous and radiopharmaceutical-carrying hollow mesoporous silica by using a combination of microfluidics and ultraviolet cross-linking. The resulting material showed uniform and controllable dimensions, excellent radiotherapeutic properties, and remarkable underwater adhesion capabilities. These characteristics help to mitigate unwanted radiation damage to non-target organs, thereby facilitating precise anti-tumour effects. Consequently, these precisely targeted radioactive HMS hold significant potential for application in the treatment of bone tumours.

Application of bioactive drugs: Emerging drugs in recent years such as cellular exosomes, biogenetic material, and genetically engineered drugs have the advantages of high targeting and specificity, lower immune response and toxicity, and better personalised design and have more room for innovation and development than the above traditional drugs.

Based on the principle that supramolecular networks formed by DNA base-pairing bind and aggregate filipin molecules and induce the construction of β-folded structures, Shen et al.[133] successfully developed hybridised filipin-DNA HMS with tunable surface stiffness, which by regulating the ratio of DNA to silk fibroin, can promote the differentiation of

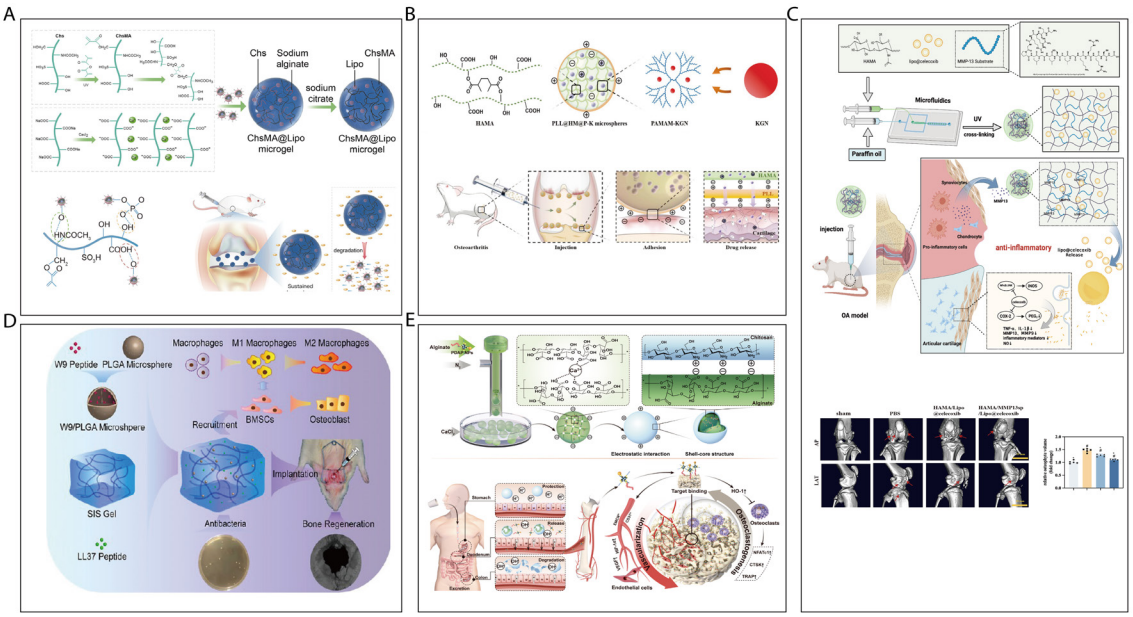

Figure 4. Different drug release characteristics of HMS in bone tissue engineering. (A) Reinforced HMS cross-linked network slow release of drugs for OA. Reprinted from He et al.[121] Copyright 2022 Acta Materialia Inc. (B) Positively charged HMS system targeted release of drugs to alleviate OA. Reprinted from Lin et al.[122] Copyright 2022 Wiley-VCH GmbH. (C) MMP13-responsive HMS targeted inflammatory environment for rapid release to alleviate OA. Scale bars: 10 mm. Reprinted from Xiang et al.[123] (D) Sequential release of drugs by the HMS system to modulate inflammation in osteogenesis. Reprinted from Ma et al.[125] Copyright 2022 American Chemical Society. (E) Shell-nucleus structure HMS system can protect and release drugs slowly for osteoporosis. Reprinted from Li et al.[128] AP: anteroposterior; Chs: chondroitin sulfate; ChsMA: chondroitin sulfate methacryloyl; COX-2: cyclooxygenase-2; CTSK: cathepsin K; EMCN: endomucin; HAMA: hyaluronic acid methacryloyl; HIF-1α: hypoxia-inducible factor 1-alpha; HO-1: heme oxygenase-1; IL-1β: interleukin-1β; iNOS: inducible nitric oxide synthase; KGN: kartogenin; LAT: lateral; Lipo: liposomes; LL37: cathelicidin LL-37; MMP: matrix metalloproteinase; N2: diatomic nitrogen; NF-κB: nuclear factor κB; NFATc1: nuclear factor of activated T-cells, cytoplasmic 1; NO: nitric oxide; NP: nanoparticle; OA: osteoarthritis; PAMAM: polyamidoamine; PBS: phosphate-buffered saline; PDAP: polyhedral oligomeric silsesquioxane/desferrioxamine@ aspartic acid 8/polyethylene glycol; PEG2: prostaglandin E2; PLGA: poly(lactic-co-glycolic acid); SIS: small intestinal submucosa; TNF-α: tumour necrosis factor-α; TRAP: tartrate-resistant acid phosphatase; UV: ultraviolet; VEGF: vascular endothelial growth factor; W9: WP9QY.

BMSCs to chondrocytes, providing an innovative strategy and material choice for cartilage regeneration and tissue engineering (**Figure 5C**). Moreover, exosomes derived from BMSCs can activate the paracrine pathway to recruit BMSCs in the damaged area and promote tissue repair. Therefore, Yang et al.[127] prepared injectable GelMA microspheres loaded with BMSC-exosomes and mixed with self-assembled peptide (KLDL-MMP1) on the basis of a microfluidic chip. Injectable enzyme-responsive KGE microspheres for accelerated vascularised bone regeneration are described in **Figure 5D**. MicroRNAs play a key role in regulating gene expression in bone regeneration and are considered new molecular targets in bone tissue engineering. Li et al.[134] modified miRNA-218 into a gene carrier polymer using a multifunctional peptide, which was encapsulated into GelMA microspheres by microfluidic technology and *in situ* photo cross-linking techniques to modulate osteoblast function and promote bone tissue regeneration (**Figure 5E**). In addition, the mitochondrial respiratory chain plays a crucial role in regulating cellular energy metabolism and oxidative stress.[135] Wang et al.[136] prepared hyaluronic acid HMS as the outer shell using microfluidics, with the inner core covalently linked to elamipretide (SS-31) peptide and WYRGRL peptide, and co-modified with resveratrol-carrying long-circulating liposomes to form the HMS system. The release of REV through the HMS system activated the SIRT3 protein, improved mitochondrial respiratory chain electron transfer efficiency, and restored mitochondrial function of chondrocytes, thereby promoting cartilage regeneration in OA.

Hydrogel microsphere cell delivery

Microspheres can be used as an efficient and effective platform for encapsulating, culturing, and delivering cells. Almost all microsphere manufacturing technologies, including emulsions, microfluidics, and electrospray are associated with cell encapsulation compatibility. As mentioned previously, microspheres can provide a large adhesion surface for cells, and the pores on their surface can facilitate diffusion and mass transfer of nutrients and oxygen. Cell delivery via HMS offers several advantages over block hydrogels. For example, the microporous gaps of the HMS ensure rapid transport and diffusion of nutrients, which facilitates cell survival and proliferation, and the HMS protects the encapsulated cells from shear damage during injection, among others.

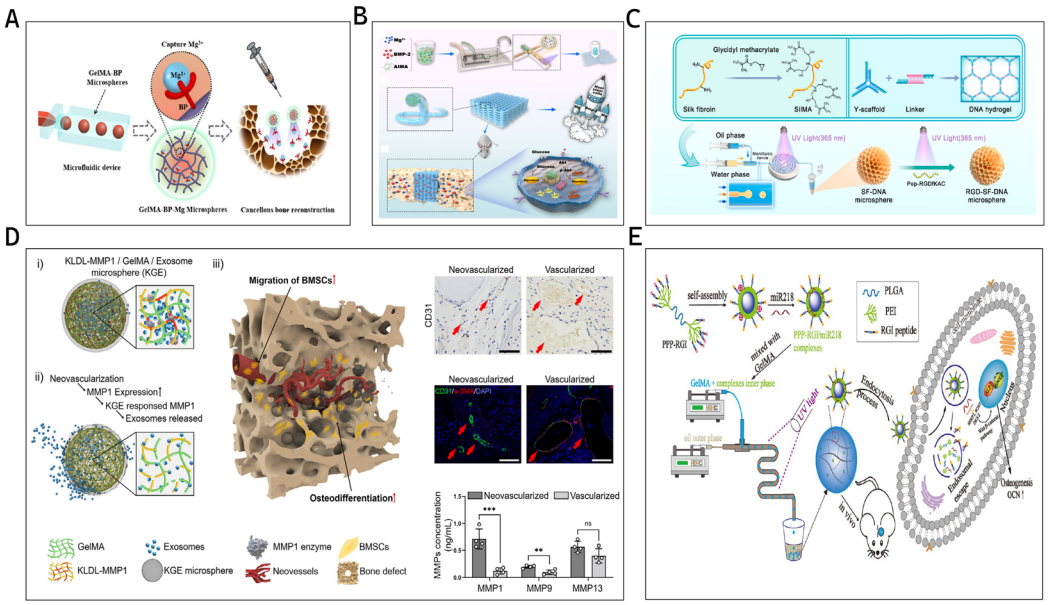

Figure 5. The HMS system promotes bone regeneration by piggybacking osteogenic active factors. (A) HMS system promotes bone regeneration by trapping magnesium ions. Reprinted from Zhao et al.[129] Copyright 2021 American Chemical Society. (B) HMS system promotes bone regeneration by releasing magnesium ions to further activate BMP-2 function. Reprinted from Lin et al.[130] (C) HMS system regulates the DNA-to-filament protein ratio to promote bone regeneration. Reprinted from Shen et al.[133] (D) HMS loads and releases BMSC-exosomes for vascularised bone regeneration. Reprinted from Yang et al.[127] (E) HMS promotes bone regeneration by loading and releasing miR-218. Reprinted from Li et al.[134] Copyright 2022 American Chemical Society. AIMA: alginate methacryloyl; Akt: protein kinase B; bFGF: basic fibroblast growth factor; BMP-2: bone morphogenetic protein-2; BMSC: bone marrow mesenchymal stem cell; BP: black phosphorus; DAPI: 4′,6-diamidino-2-phenylindole; ECM: extracellular matrix; GelMA: gelatine methacrylate; GMS: gelatin methacrylate microspheres; HMS: hydrogel microsphere; KLDL: Ac-KLDLKLDLVPMSMRGGKLDLKLDL-CONH2 pedtide ns: not significant; Mg^{2+}: magnesium ion; MMP: matrix metalloproteinase; NP: nucleus pulposus; ns: not significant; OCN: osteocalcin; p-Akt: phosphorylated protein kinase B; PEI: polyethyleneimine; Pep-RGDfKAC: peptide sequence of Arg-Gly-Asp with an acryloyl group; PLGA: poly(lactic-co-glycolic acid); PPP: poly(lactide-co-glycolide)-g-polyethylenimine-b-polyethy-lene; RGD: Arg-Gly-Asp peptide; RGI: Arg-Gly-Iso peptide; RUNX2: Runt-related transcription factor 2; SF-DNA: silk fibroin-DNA; SilMA: silk fibroin methacryloyl; UV: ultraviolet; α-SMA: α-smooth muscle actin.

Hydrogel microsphere loaded 'cell-on-cell' system

Depending on their application, cells can be inoculated on a surface or encapsulated within microspheres. Inoculation of cells on the surface of microspheres, also known as 'cell-on-cell' systems, is commonly used for *in vitro* expansion of cells.[12, 137] The system can be cultured *in vitro* prior to delivery and supports cell adhesion, proliferation, and differentiation through different surface modifications. Compared with traditional two-dimensional cell culture, the microcarriers have a higher surface area-to-volume ratio, providing more space for cell expansion in culture, while the 3D structure of the microspheres themselves can better promote cell adhesion and proliferation.[138] Simple changes in cell culture conditions from plates to microspheres have been reported to promote osteogenesis in a variety of MSCs, and researchers have compared two-dimensional and 3D cultures of BMSCs with GMs for MSCs. The results showed that the bone marrow stromal cells attached to the surface of the GMs and stayed viable in the 3D culture. On tissue culture plates (BMSCs-tricalcium phosphate), BMSCs-GM proliferated faster than BMSCs and exhibited higher stem cell characteristics. In addition, GM enhanced the efficiency of cartilage formation of BMSCs *in vitro* (**Figure 6A**).[139] Meanwhile, mechanical signals on the surface of HMS can also regulate cell differentiation fate, for example, fibronectin-modified GelMA microspheres were prepared by Chen et al.[140] Nucleus pulposus-like differentiation of intervertebral disc progenitor/stem cells were induced by varying their elastic modulus (1–10 kPa) and ligand density (2–10 μg/mL) (**Figure 6B**). Moreover, when the HMS has a rough surface and porous structure, cell adhesion and proliferation can be enhanced by contact-directed effects. Nanomodification enables microspheres to benefit from the size effect of nanomaterials, which promotes specific cell-material interactions and enhances the biological properties of the materials. Researchers successfully prepared mineralised ALG microspheres by forming a HA coating on the surface of ALG microspheres using Dulbecco's modified Eagle's medium. The experimental results verified that the HMS exhibited good biocompatibility and osteogenic properties, and the HA coating also contributed to the active migration of osteoblasts to the surface of mineralized ALG microspheres.[141] In addition, the porous structure provides sufficient internal space for inward cell growth and protects the cells during implantation. The pore size and porosity of microspheres are considered the key factors for cell loading. Porous HMS has greater specific surface area and solute diffusivity than HMS, which improves more attachment points for cells. Yuan et al.[142] prepared porous shape memory cryogel microspheres

(CMS) by GelMA. The pore size of CMS was adjusted by a gradient cooling program, and a 30-minute gradient cooling variant (CMS-30) was used to achieve an optimal pore size of 15.5 ± 6.0 μm, which showed better adhesion and proliferation ability for human bone marrow stromal cells and human umbilical vein endothelial cells. In *in vitro* experiments, CMS also exhibited high levels of osteocalcin and CD31 for vascular bone-like tissue development (**Figure 6C**).

Hydrogel microsphere-loaded 'intracellular' system

The term 'intracellular' system refers to microspheres that encapsulate cells. This system typically delivers cells immediately after encapsulation, which provides a protective environment for the encapsulated cells to ensure their survival and proliferation.[12, 144] In this system, the nature of the raw material, diameter, porosity of the structure, and degradation of the microspheres are the main factors that affect cell migration and proliferation. The diameter and porosity of microspheres are closely related to oxygen transport and nutrient metabolism. The system can support cell migration and proliferation within the microspheres as well as from the interior to the surface while maintaining stem cell viability. The most common intracellular strategy is HMS delivery of cells alone, e.g. GelMA microspheres piggybacked with BMSCs for bone regeneration.[145] In addition, HMS-loaded cells are also optimised in the following manner: growth factors are added to enhance the biological functions of cells, while HMS delivers to the cells. Zhang et al.[146] fabricated injectable hybrid RGD-ALG/synthesised lithium saponin HMS by microfluidic device. These microspheres co-capsulated human dental pulp stem cells and VEGF. After delivery, these cells proliferated and differentiated in response to the slow release of VEGF, which significantly promoted the regeneration of pulp-like tissues as well as the formation of new microvessels. By piggybacking many different cells through microspheres to create a multifunctional ECM, tissue engineering focuses on creating the vascular network and ECM required for cell survival. To this end, Zhang et al.[143] prepared osteoid collagen microspheres by inoculating human umbilical vein endothelial cells onto collagen microspheres harbouring human osteoblast-like cells (MG 63) and collagenase. Vessel-like channels were formed between these microspheres that formed naturally through sphere stacking and gelatine degradation. Ultimately, these channels promoted vascularised osteogenesis within the matrix containing osteoblasts (**Figure 6D**).

Bone regeneration hydrogel microsphere scaffolds

Microspheres can also be used as building blocks for macroscopic scaffolds, which can further localise microspheres to the desired location. These scaffolds are typically implanted as temporary space fillers into bone defects to provide initial mechanical support in the early stages of bone healing. Meanwhile, the gap space between microspheres is sufficient to allow oxygen and nutrients to enter, supporting the inward growth of cells.[147, 148] At present, there are two main types of bone defect scaffolds commonly used, namely scaffolds with microsphere binding and scaffolds based on microsphere construction.

Utilisation of hydrogel microsphere composite stents

HMS composite scaffolds are constructs of scaffolds that use microspheres as discrete components embedded in a continuous matrix. The continuous matrix can be a polymer or a ceramic. The mechanical properties of composite scaffolds are usually determined by the continuous matrix. However, when the microsphere concentration is significant, the size and density of the microspheres also affect the mechanical properties of the scaffold.[12, 149] Simple incorporation of biomolecules into native scaffolds leads to denaturation of these molecules by exposure to harsh scaffold fabrication conditions, acidic degradation products, and the hydrophobic surface of the scaffold; therefore, microsphere-bound scaffolds are very friendly to some environmentally sensitive biomolecules. In addition, the properties of the microspheres and the continuous matrix in microsphere-conjugated scaffolds can be simultaneously modulated to optimise the release pattern of bioactive factors. For example, during bone regeneration, angiogenesis precedes bone regeneration to provide blood supply. Therefore Han et al.[150] designed a new injectable dual-drug program-releasing chitosan nanofiber microsphere-based poly(D,L-lactic-co-glycolic acid)-b-polyethylene glycol-b-poly(D,L-lactic-co-glycolic acid) hydrogel to achieve the release of microspheres-loaded VEGF and dental pulp stem cell-derived exosomes. Rapid release of VEGF promotes rapid initiation of angiogenesis, while dental pulp stem cell-derived exosomes release ensures sustained osteogenesis (**Figure 7A**). For bone regeneration, osteoconductivity and sufficient calcium-phosphate nucleation sites are essential. Davis et al.[151] designed a composite hydrogel encapsulating calcium-phosphate-modified poly (propylidene-co-ethylene-glycolide) HMS and MSCs. Calcium-phosphate, as an osteoconductivity-promoting ingredient, provides mechanistic guidance to facilitate the MSCs' osteogenic process.

Utilisation of hydrogel microsphere scaffolding

According to the bottom-up construction strategy, microspheres can be used as the building blocks for scaffold fabrication, and scaffold construction can be carried out based on microspheres. This scaffold type supports inward cell growth, while the gaps present between the microspheres are sufficient to allow oxygen and nutrients to enter. Randomised stacked scaffolds combine the advantages of controlled release of microspheres with the ability of porous scaffolds for cell adhesion.[152] Chen et al.[153] fabricated macroscopic bone constructs by inoculating human amniotic membrane MSCs with porcine skin gelatine as carriers in micromanipulation vials, where the cell-carrying microspheres were packaged in micromanipulation vials in reaction for a certain period of time to produce the formation of a dense ECM by holding the microspheres together. In another strategy, the microspheres are fused into interconnected microporous structures using a sintering method. Several sintering methods such as solvent-based sintering, heat sintering, and selective laser sintering have been developed to fabricate scaffolds.[142] Sintered microsphere-based scaffolds typically exhibit excellent mechanical properties, controllable pore size and porosity, and high pore interconnectivity[154, 155] but inadequate sintering can lead to poor mechanical and structural integrity, and excessive

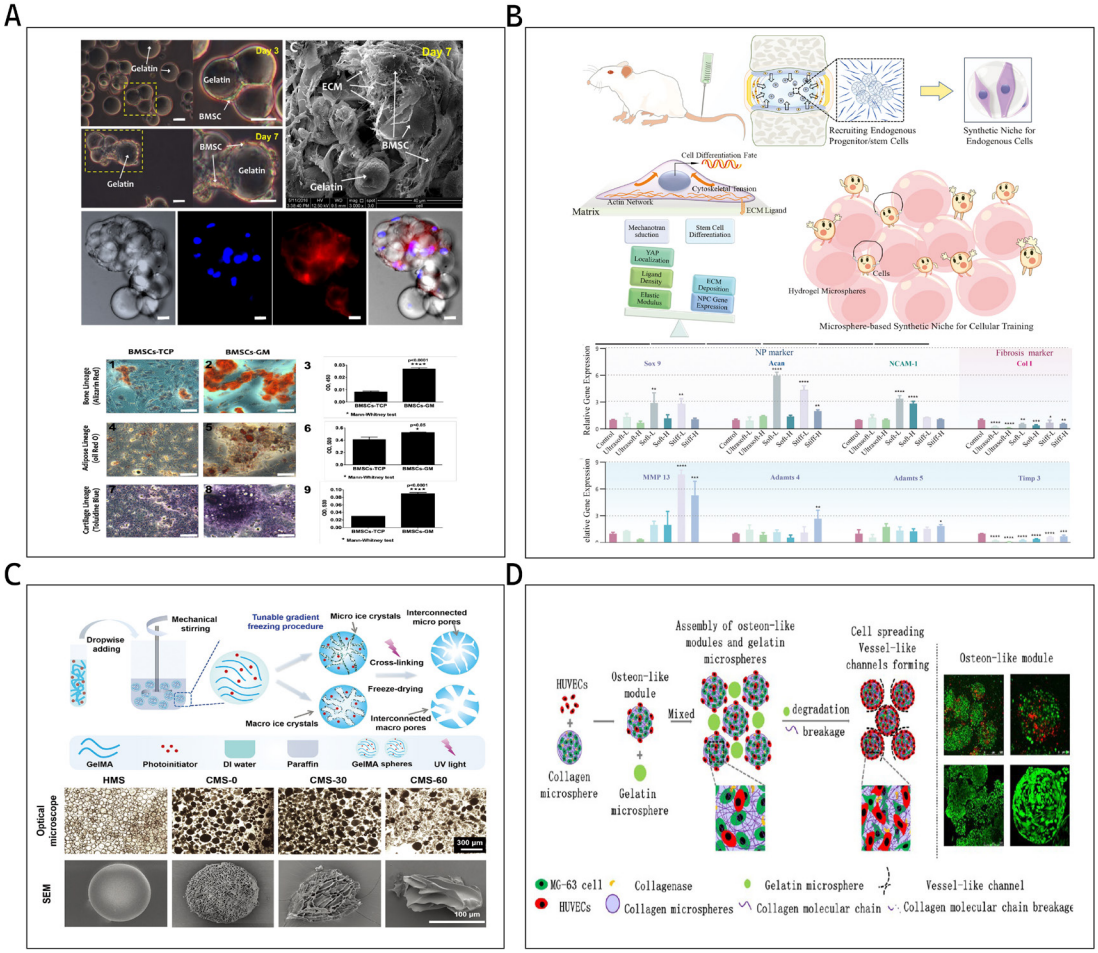

Figure 6. HMS and cell co-culture system. (A) 3D structure of HMS surface promotes the proliferation and differentiation of BMSCs. Reprinted from Sulaiman et al.[139] (B) Modulation of elastic modulus of HMS surface induces the differentiation of intervertebral disc progenitor cells. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Reprinted from Chen et al.[140] Copyright 2023 Wiley-VCH GmbH. (C) Modulation of the porosity of HMS surface enhances the proliferation and adhesion ability of BMSCs and HUVECs. Reprinted from Yuan et al.[142] Copyright 2021 Wiley-VCH GmbH. (D) Promotion of vascularised bone regeneration by piggybacking BMSCs and HUVECs in and on HMS, respectively. Reprinted from Zhong et al.[143] Copyright 2017 American Chemical Society. Acan: aggrecan; Adamts: a disintegrin and metalloproteinase with thrombospondin motifs; bFGF: basic fibroblast growth factor; BMSC: bone marrow mesenchymal stem cell; CMS: cryogel microsphere; Col 1: collagen I; DI: deionized; ECM: extracellular matrix; GelMA: gelatine methacrylate; GM: gelatine microsphere; HMS: hydrogel microsphere; HUVEC: human umbilical vein endothelial cell; MMP 13: matrix metalloproteinase-13; NCAM-1: neural cell adhesion molecule 1; NP: nucleus pulposus; NPC: nucleus pulposus cells; OD: optical density; SEM: scanning electron microscope; Soft-H: group of high modulus of elasticity and high ligand density; Soft-L: group of low modulus of elasticity and low ligand density; Stiff-H: group of high modulus of elasticity and high ligand density; Stiff-L: group of high modulus of elasticity and low ligand density; Timp3: tissue inhibitor of metalloproteinases 3; Ultrasoft-H: group of very low modulus of elasticity and high ligand density; Ultrasoft-L: group of very low modulus of elasticity and low ligand density; UV: ultraviolet; YAP: yes-associated protein.

sintering can lead to pore closure. In addition, microspheres can be assembled into complete scaffolds by introducing physical cohesive forces (e.g. electrostatic forces). Luo et al.[156] prepared a new charge-driven self-assembled microsphere hydrogel scaffold based on charge interaction. It combines positively charged chitosan methacrylate microspheres containing black phosphorus and negatively charged hyaluronic acid methacrylyl microspheres containing basic fibroblast growth factor (bFGF) through electrostatic attraction (**Figure 7B**). 3D printing of microspheres is an emerging technology that can be used to rapidly fabricate macroscopic scaffolds with complex structures by accurately accumulating microspheres with the assistance of a computer. Seymour et al.[157] created HMS scaffolds with varying porosities by varying the ratio of GelMA-to-gelatine while keeping the total concentration constant at 6 wt%, which allowed for tuning the void ratio of the printed scaffolds from 0.20 to 0.57. In addition, human umbilical vein endothelial cells inoculated onto the printed structures were observed to migrate into the HMS scaffolds in a void ratio-dependent manner (**Figure 7C**).

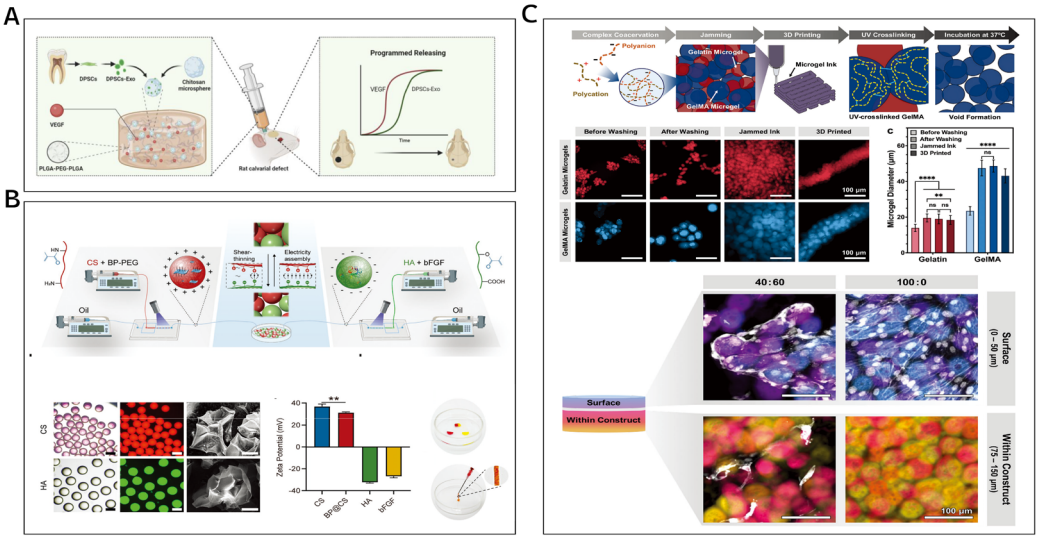

Figure 7. HMS scaffolds. (A) composite PLGA-PEG-PLGA microsphere scaffolds piggybacking VEGF with DPSC-exosomes to promote vascularised osteogenesis. Reprinted from Han et al.[150] Copyright 2023 Elsevier B.V. (B) Electrostatically self-assembled BP/CS microsphere scaffolds. Reprinted from Luo et al.[156] Copyright 2023 Wiley-VCH GmbH. (C) 3D printed Gel/GelMA microsphere scaffold. Reprinted from Seymour et al.[157] Copyright 2021 Wiley-VCH GmbH. 3D: three-dimensional; bFGF: basic fibroblast growth factor; BP: bisphosphonate; CS: chitosan methacrylate; DPSC: dental pulp stem cell; Exo: exosome; Gel: gelatine; GelMA: gelatine methacrylate; HA: hyaluronic acid; HMS: hydrogel microsphere; ns: not significant; PEG: polyethylene glycol; PLGA: poly(lactic-co-glycolic acid); UV: ultraviolet; VEGF: vascular endothelial growth factor.

Hydrogel Microsphere Regulates the Microenvironmental Mechanism of Bone Regeneration

Hydrogel microsphere system regulates the circulatory microenvironment

HMS can be designed as a drug-carrying system for the release of growth factors to promote blood vessel formation in endothelial cells. By carrying growth factors inside or on the surface of HMS, controlled release of growth factors can be achieved to effectively regulate endothelial cell growth and angiogenesis. For example, Shin et al.[158] doped porous PLGA microspheres loaded with VEGF into an ALG hydrogel containing the anti-apoptotic agent TAT-heat shock protein 27 to prepare a microsphere/hydrogel hybrid delivery system. Sequential *in vitro* release of TAT-heat shock protein 27 and VEGF was achieved by the hybrid system to promote neovascularisation. The binding of VEGF to the endothelial cell receptor VEGFR activated the PI3K/Akt pathway, mTOR pathway, and signal transducers and activators of transcription 3 pathway, which are intertwined to regulate endothelial cell function and blood vessel formation.[159-161] In addition, Liu et al.[162] promoted burned skin regeneration by designing ALG microspheres with bFGF. bFGF induced activation of the FGFR downstream pathway further promoted angiogenesis. These growth factors can promote angiogenesis, osteoblast proliferation, and differentiation and regulate the synthesis and deposition of bone matrix, thus promoting vascularised osteogenesis.

HMS has a specific structure and porosity that provides the support and space needed for endothelial cell growth. Endothelial cells can attach to the surface or inside of the HMS and grow along the structure of the microspheres, mimicking the process of real blood vessel formation.[163] Meanwhile, HMS has certain mechanical properties that can provide stable support for neovascularisation and help maintain the structural integrity of blood vessels. This mechanical support plays an important role in the formation and function of blood vessels. Meanwhile, the newborn blood vessels not only provide a channel for the supply of oxygen and nutrients but also play a role in guiding and attracting the surrounding osteoblasts.

Hydrogel microsphere regulates the immune environment in bone regeneration

HMS regulates the local inflammatory microenvironment in bone regulation, which includes two aspects, namely the maintenance of redox homeostasis as well as the modulation of immune cell behaviour and function.

Hydrogel microsphere regulates redox homeostasis in bone regeneration

Enhanced inflammation and oxidative stress are present in damaged bone sites.[164] The current strategy is to functionalise HMS using antioxidants, metal ions, or other biomaterials (e.g. nano-enzymes) to scavenge ROS, enhance the expression of anti-inflammatory genes, and restore redox homeostasis to provide a microenvironment conducive to the proliferation

and osteogenic differentiation of stem cells. For example, Zheng et al.[165] prepared hydrogen ion trapping HMS (GMNP) by mineralised TGF-β and catalase nanoparticles. Catalase inhibited the over-activation of the thioredoxin-interacting protein (TXNIP)/NOD-like receptor protein 3 (NLRP3)/IL-1β cascade axis, suppressed the production of ROS, and neutralised the acidic microenvironment by trapping excess hydrogen ions through the calcium carbonate mineralised layer (**Figure 8A**). Redox disturbances as well as microenvironmental acid-base imbalances exist during bone regeneration, and the use of HMS to regulate the pH in the bone regeneration environment is a promising approach. Under different pH conditions, cells may exhibit different activities and functions. By regulating the pH of the bone regeneration environment, cell proliferation, differentiation, and matrix deposition can be influenced, which in turn regulates the regeneration rate and quality of bone tissues. pH-responsive HMS allows microspheres to release or take up specific substances to regulate the pH of the surroundings when the environmental pH changes. However, currently pH-responsive HMS is often applied to anti-tumour drugs and in case of wound infections. pH-responsive HMS applied to regulate the pH microenvironment for bone regeneration needs to be further researched and developed.

Hydrogel microsphere regulates immune cell behaviour in bone regeneration

Macrophages and neutrophils are the main immune cells involved in the early inflammatory response to bone healing. Neutrophils clear dead cells and secrete chemokines and cytokines to recruit macrophages; macrophages phagocytose necrotic cells and cellular debris at the site of bone injury and differentiate into osteoclasts to perform bone resorption. Macrophages can also be polarised into pro-inflammatory M1 and anti-inflammatory M2, and M1 macrophages can regulate the differentiation of MSCs into osteoblasts through the intervention of the cyclooxygenase-2-prostaglandin E2 pathway at the initial stage of inflammation. Osteoblast differentiation and M2 macrophages promote collagen deposition and restore tissue homeostasis through the production of anti-inflammatory factors.[166] Sun et al.[167] fabricated GelMA microspheres using microfluidic technology and inserted BP-conducting nanosheets into a hydrogel matrix. Through electrical stimulation, dental pulp stem cells released cytokines. These cytokines could upregulate the expression of M2-related genes such as *FABP3*, *A2M*, and *EPHB1*, thereby improving the inflammatory microenvironment of bone regeneration (**Figure 8B**). In addition, Li et al.[168] validated the use of gelatine-heparin microspheres containing TGF-β1 loaded onto injectable lithium heparin hydrogel for regulation of bone regeneration. Release of TGF-β1 guides macrophages to undergo M2 polarisation and express signals such as SMAD2 (small mother against decapentaplegic homolog 2), SMAD3 (small mother against decapentaplegic homolog 3), AKT, Snail, VEGF, and BMP-2 factors. M2 macrophage polarisation promotes osteoblast osteogenic expression, thereby directing bone regeneration (**Figure 8C**). The mechanisms of M2 macrophages in promoting osteogenesis are multifaceted. They modulate the inflammatory response, reduce the levels of inflammatory mediators, and secrete a variety of growth factors including TGF-β, VEGF, and bFGF. These growth factors have a promoting effect on the proliferation, differentiation, and matrix synthesis of osteoblasts, which contribute to the regeneration and repair of bone tissue. In addition, M2 macrophages can secrete MMPs and tissue inhibitors of metalloproteinases to regulate the balance of matrix degradation and synthesis, thereby promoting the formation of new bone tissue.[169] Although existing studies have achieved better bone repair by regulating macrophages, it is not clear whether this process induces macrophage polarisation to other sub-phenotypes and how these sub-phenotypes affect bone repair. Some studies have shown that other immune cells such as dendritic cells and different subtypes of T cells can improve the inflammatory environment of bone regeneration and promote bone regeneration through cellular burial and other actions. However, at present, there is no report on the use of the HMS system to interact with these cells to improve the microenvironment of bone regeneration, and its application needs to be further explored.[170]

Hydrogel microsphere system promotes the mechanism of neuralised osteogenesis

With the development and application of microspheres, researchers are increasingly focusing on the use of microspheres to deliver cells and functional factors to repair the damaged nervous system. Currently, cell transplantation is considered one of the most promising methods for nerve repair. Neural precursors, stem cells, MSCs, and Schwann cells are widely used for nerve repair. The main goals of stem cell therapy are to regenerate axons, prevent apoptosis, and replace lost cells. Shendi et al.[171] fabricated vinyl sulfone-modified HA microspheres encapsulated with neural stem cells to promote CNS repair by using delivered neural cells. However, there is no report on the use of HMS for direct delivery of neuronal cells to promote neuralised osteogenesis.

Among the biomaterials used for nerve repair, many biodegradable synthetic polymers derived from glycolic acid, lactic acid, and caprolactone have been used to fabricate microsphere-based delivery systems, whereas further encapsulation of growth factors through the use of HMS can reduce the rate of growth factor release and prolong the duration of *in vivo* activity. Li et al.[172] fabricated a GM composite RIBQ, RGD (Arg-Gly-Asp peptide) peptide, IK19 (Ac-CSRARKQAASIKVAVSADR-NH_2) peptide, BFP-1 (bone-forming peptide-1) peptide, and QK (Ac-KLTWQELYQLKYKGI-NH_2) peptide to promote neurovascularised bone regeneration. Although the detailed mechanism of the IK19 peptide's action is still under investigation, it was verified that it could promote neuronal survival, protection, and repair by regulating neurotrophic factors and modulating the inhibitory pathway of neuronal apoptosis (**Figure 9A**). Meanwhile Chen et al.[173] prepared a bFGF-loaded GelMA HMS. Utilising the porous structure of GelMA microspheres and easy chemical modification, bFGF could be readily loaded and gradually released. By co-culturing with neural stem cells, it was verified that the bFGF-loaded GelMA microspheres could effectively promote the proliferation and differentiation of neural stem cells (**Figure**

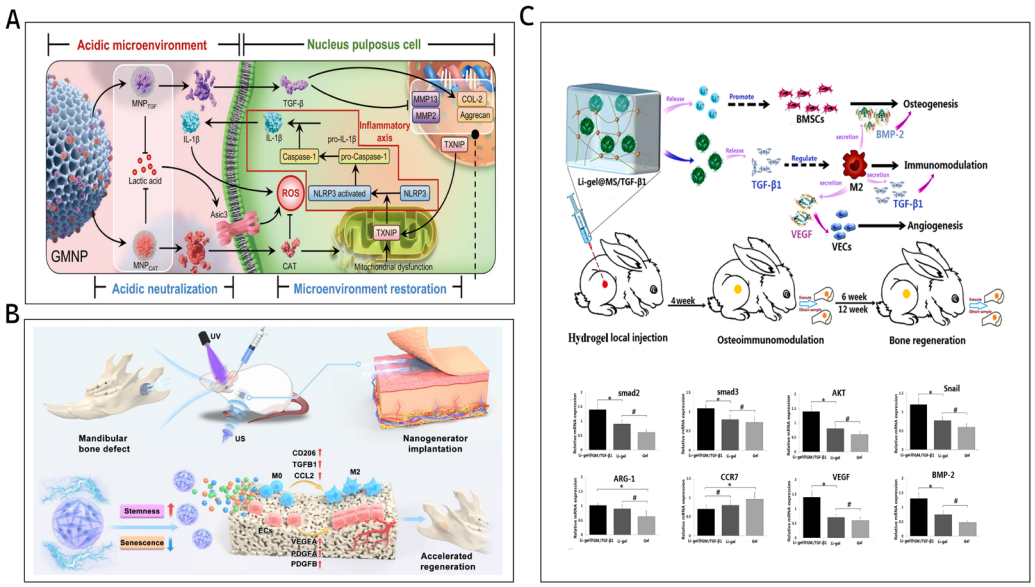

Figure 8. HMS modulates the immune microenvironment of bone regeneration. (A) GMNP microspheres inhibit ROS and improve the inflammatory microenvironment. Reprinted from Zheng et al.[165] Copyright 2023 Wiley-VCH GmbH. (B) BP/GelMA microspheres induce M2 macrophage polarisation by electrical stimulation to modulate the immune microenvironment. Reprinted from Sun et al.[167] Copyright 2023 Wiley-VCH GmbH. (C) Li-gel/MS/TGF-β1 microspheres induce M2 macrophage polarisation to modulate the immune microenvironment. Reprinted from Li et al.[168] Copyright 2022 Published by Elsevier B.V. *$P < 0.05$, #$P < 0.05$. AKT: protein kinase B; ARG-1: arginase-1; BMP: bone morphogenetic protein; BMSC: bone marrow stromal cell; BP: bisphosphonate; CAT: catalase; CCR7: chemokine receptor 7; COL-2: collagen II; EC: endothelial cell; GelMA: gelatine methacrylate; GMNP: hydrogen ion-capturing hydrogel microspheres; HMS: hydrogel microsphere; IL-1β: interleukin-1beta; Li-gel: lithium heparin hydrogel; MMP: matrix metalloproteinase; MNP_{CAT}: catalase-loaded mineralised nanoparticles; MS: microsphere; NLRP: NOD-like receptor protein; PDGF: platelet-derived growth factor; ROS: reactive oxygen species; smad: small mother against decapentaplegic homolog; TGF-β: transforming growth factor β; TXNIP: thioredoxin-interacting protein; US: ultrasound; VEC: vascular endothelial cell; VEGF: vascular endothelial growth factor.

9B). Different biomaterials and fabrication methods confer strong adaptability to meet certain requirements of neural repair. As mentioned above, HMS, with its good injectability and designability, has very promising applications in nerve repair. The newly repaired nervous system can regulate osteogenesis through neurotransmitter regulation, neurogenic active factors, bio-signalling molecules, and neuroendocrine regulation. For instance, neuronal cells produce CGRP, which activates intracellular cyclic adenosine monophosphate and downstream signalling pathways in osteoblasts through its CGRP receptor complexes and promotes osteoblasts' adhesion to biomaterials. In addition, CGRP can activate ERK1/2-mitogen-activated protein kinase, Wnt/β-catenin, nuclear factor kappa B, and other signalling pathways, regulate the function of osteoblasts through a variety of signalling pathways, and provide potential therapeutic significance for bone diseases.[174] Besides, vasoactive intestinal peptide, neurocyte exosomes, norepinephrine, and other biologically active substances work together to regulate bone metabolism.[175] However, the use of HMS system to promote the regeneration of neuralised bone is still less well reported.

Hydrogel microsphere system regulates functional cells of the bone lineage

As a carrier material with good biocompatibility and controlled release, HMS is widely used to carry metal ions, hormones, bioactive factors, and cellular active substances to promote bone regeneration. Through appropriate design and preparation process, these substances can be encapsulated and released in the HMS, thus realising their precise controlled release during the treatment process and promoting bone healing. For example, HMS can be used as a carrier to release metal ions by adsorption or ion exchange. HMS can carry metals such as magnesium and cerium to promote bone regeneration, and studies have shown that magnesium can stimulate the osteogenic differentiation and mineralisation of MSCs through the activation of Notch1 signalling. In addition, it can activate the classical Wnt signalling pathway in human BMSCs, and increase the bioactivity of BMP by enhancing BMP receptor recognition and SMAD signalling pathway, thus guiding its differentiation towards the osteoblast lineage.[176, 177] In addition, the trace element cerium ion has been shown to promote bone regeneration in some studies. Liu et al.[178] prepared GelMA microspheres by microfluidic equipment and doped cerium ions into them. The results showed that cerium ion-containing HMS may induce the activation of the Wnt/β-catenin signalling pathway, thereby accelerating the process of bone regeneration. The activation of the Wnt/β-catenin pathway promoted the expression of the downstream osteogenic genes *COL-1*, *RUNX 2*, and osteocalcin, and stimulated the secretion of VEGF from the BMSCs, which regulated the osteogenic differentiation and angiogenesis for vascularised

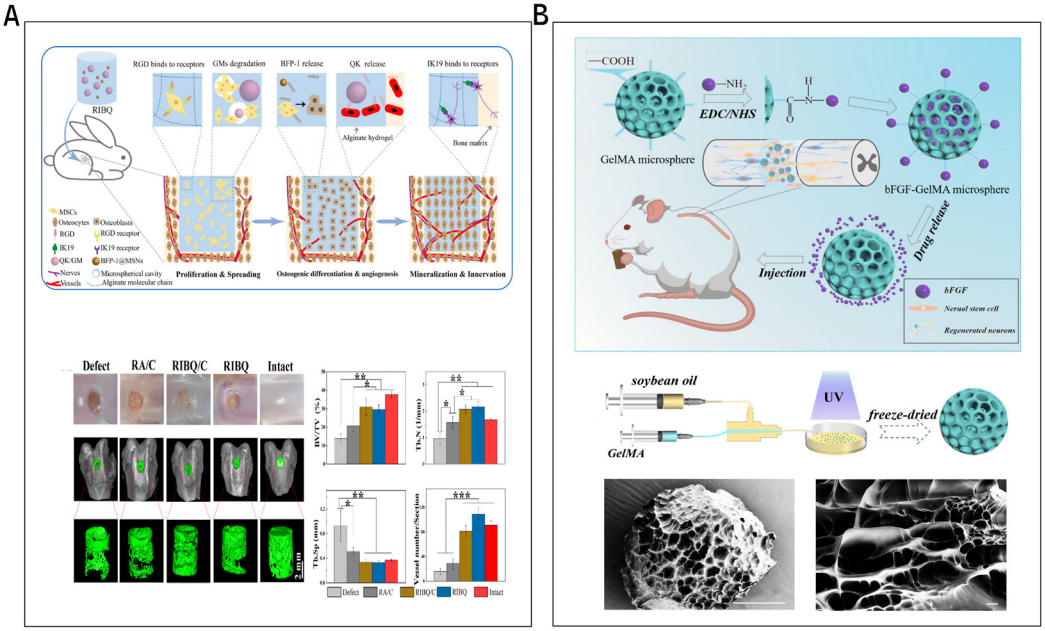

Figure 9. The HMS system promotes nerve regeneration. (A) RIBQ composite microspheres promote neural-vascularised osteogenesis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Reprinted from Li et al.[172] Copyright 2023 Royal Society of Chemistry. (B) bFGF/GelMA microspheres promote proliferation and differentiation of neural stem cells. Reprinted from Chen et al.[173] bFGF: basic fibroblast growth factor; BFP-1: bone-forming peptide-1; BV/TV: bone volume to total volume; EDC/NHS: N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride/N-hydroxy succinimide; GelMA: gelatine methacrylate; GM: gelatine microsphere; IK19: Ac-CSRARKQAASIKVAVSADR-NH_2 peptide; MSC: mesenchymal stem cell; MSN: mesoporous silica nanoparticles; QK: Ac-KLTWQELYQLKYKGI-NH_2 peptide; QK/GM: QK-loaded gelatin microspheres; RA/C: cell-laden and RGD peptide grafted alginate hydrogel; RGD: Arg-Gly-Asp peptide; RIBQ: hydrogel–microsphere composites; Tb.N: trabecular number; Tb.Sp: trabecular separation; UV; ultraviolet.

bone regeneration (**Figure 10A**). Moreover, HMS can be used to piggyback on hormones and control the rate and dose of hormone release by modulating the structure and composition of the hydrogel to achieve a sustained and controlled release of hormones. Dexamethasone's injectable self-healing HMS can be used for bone regeneration,[179] which can pass through the cell membrane into the cell and interact with the glucocorticoid receptor in the cell's nucleus. The glucocorticoid receptor in the cell nucleus upon forming a dexamethasone-glucocorticoid receptor complex can further regulate the downstream pathways of bone metabolism and promote osteogenesis, but long-term or excessive use of dexamethasone may lead to adverse effects such as osteoporosis. The role of hormones in osteogenesis is complex and two-faceted, depending on the specific hormone type, dosage, and mode of administration; however, the exact mechanisms involved remain to be investigated.[180-182] In addition, HMS is often used to piggyback bioactive factors such as growth factors and cytokines to promote tissue regeneration and repair. These factors can be stably preserved by HMS and progressively released as needed to enhance the therapeutic effects. As mentioned, HMS carries BMP-2, BMP-2 binds to the receptor and activates the type I receptor, which phosphorylates SMAD1/5/8 proteins, and the phosphorylated SMAD1/5/8 proteins bind to SMAD4 proteins to form a complex, which enters the cell's nucleus and regulates the transcriptional activity of genes, including those related to osteoclast differentiation and osteogenesis, such as alkaline phosphatase and osteocalcin.[183] In addition, BMP-2 can affect osteogenesis through non-SMAD signalling pathways, including activation of mitogen-activated protein kinase (e.g. ERK1/2, c-Jun N-terminal kinase, and p38 signalling pathways) to participate in cell proliferation, differentiation, and matrix synthesis, as well as activation of PI3K/Akt to promote cell survival and proliferation, which ultimately affects osteoblast function and bone tissue production.[184] HMS can also act as an effective carrier of cellular activators, which helps cell survival and bone tissue production. Furthermore, it can serve as an effective carrier of cellular active substances to help cell survival and proliferation. In tissue engineering and regenerative medicine, this design can help cells survive and proliferate better after implantation or transplantation and promote tissue repair and regeneration. Pan et al.[185] promoted bone regeneration by using HMS to piggyback exosomes from BMSCs containing microRNA-29a (**Figure 10B**). MiR-29a promotes bone regeneration by inhibiting the expression of genes associated with bone resorption such as MMP-2 and MMP-9.[186] At the same time, upregulation of miR-29a enhances the activity of BMP signalling pathway and promotes the expression of osteogenesis-related genes (e.g. collagen I, osteocalcin). In addition, miR-29a has been shown to play a role in mitochondrial autophagy and interact with other molecules such as circHIPK3 (circular homeodomain-interacting protein kinase 3) and PINK1 (PTEN-induced kinase 1) to promote osteogenic differentiation.[187]

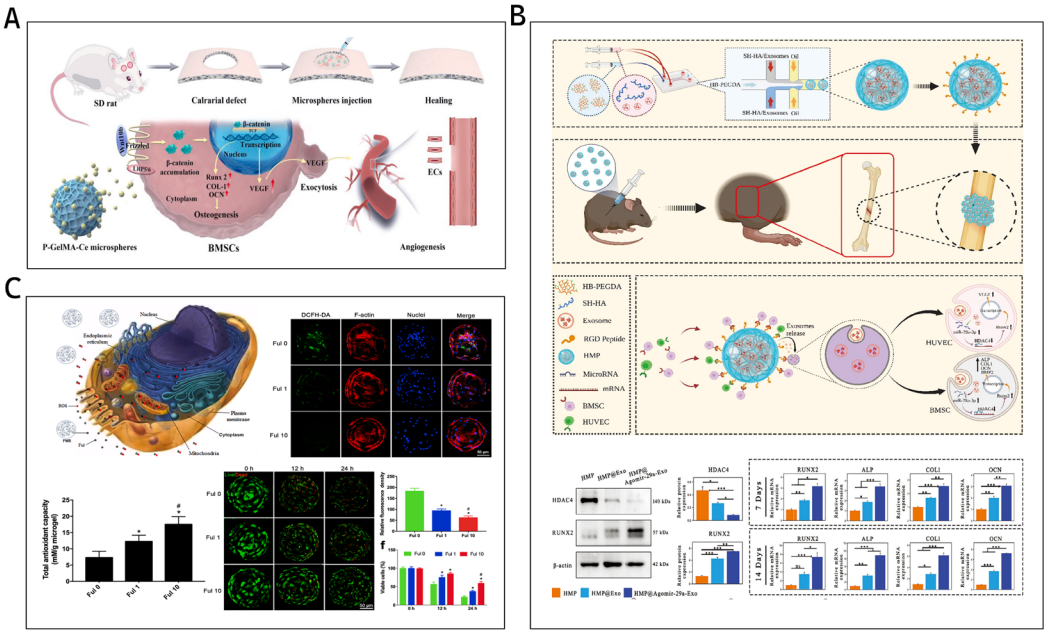

Figure 10. The HMS system regulates functional cells of the bone lineage. (A) Cerium-ion loaded GelMA microspheres activate the Wnt/β-catenin pathway to promote bone regeneration. Reprinted from Liu et al.[178] (B) HMS system loaded with microRNA-29a activates the BMP pathway to promote vascularised bone regeneration. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Reprinted from Pan et al.[185] Copyright 2023 Wiley-VCH GmbH. (C) Microspheres promote bone regeneration by restoring the redox homeostasis of BMSCs. Reprinted from Yang et al.[189] $*P < 0.05$, *vs.* Ful 0 group; $\#P < 0.05$, *vs.* Ful 10 group. ALP: alkaline phosphatase; BMP: bone morphogenetic protein; BMSC: bone marrow mesenchymal stem cell; COL1/COL-1: collagen I; DCFH-DA: 2′,7′-dichlorodihydrofluorescein diacetate; EC: endothelial cell; Exo: exosome; FMS: fullerol-hydrogel microfluidic sphere; Ful: fullerol; GelMA: gelatine methacrylate; HB-PEGDA: hyperbranched poly ethylene glycol diacrylate; HDAC4: histone deacetylase 4; HMP: hydrogel microparticle; HMS: hydrogel microsphere; HUVEC: human umbilical vein endothelial cell; LRP5/6: low-density lipoprotein receptor-related protein 5/6; ns: not significant; OCN: osteocalcin; P-GelMA-Ce: phosphorylated gelatin methacrylamide-cerium composite microspheres; RGD: Arg-Gly-Asp; Runx2: Runt-related transcription factor 2; SD: Sprague-Dawley; SH-HA: sulfhydryl-modified hyaluronic acid; TCF: T-cell factor; VEGF: vascular endothelial growth factor.

Hydrogel microsphere modulates the physicochemical bone regeneration microenvironment

Hydrogel microsphere system incorporates physical cues to promote bone regeneration

HMS can also work synergistically with external physical stimuli such as mechanical force, NIR, ultrasound, and electrical stimulation. HMS can be assembled into scaffolding structures to provide the mechanical support required for cell growth and migration. Such scaffold structures can mimic the microenvironment of natural bone tissue, providing the appropriate growth space and support for cells that can help promote cell adhesion, proliferation, and directed growth.[188] Functionalised microgels provide the ability to easily incorporate bioactive cues into the scaffold. The microgel modulus can be varied to control the cellular response within the assembled scaffold. The role of matrix mechanics in controlling cell fate and function is well-known in bulk hydrogel scaffolds. For example, microspheres of hydroxyapatite-modified hydrogels facilitated force transmission by mimicking the high mechanical strength of bone regeneration ECM and further activated signalling pathways such as Wnt/β-catenin and PI3K/Akt promoting the proliferation of BMSCs and differentiation into bone. In addition, when endothelial cells are exposed to tension, they release VEGF, which enhances the proliferation of endothelial cells and the formation of H-shaped blood vessels and provides oxygen and nutrients to the bone tissue, thus promoting bone regeneration. Researchers often use NIR light-responsive HMS to control the slow release of drugs. Low-intensity NIR light also shows a strong potential to promote tissue regeneration, and it has been shown that NIR stimulation promotes osteoblastic differentiation of BMSCs and MC3T3-E1 cells, which is related to the presence of the core bio-clastogenic protein CRY1 in the cell nucleus. CRY1 increased ubiquitination in the nucleus,[56] but the specific signalling pathway remains to be further investigated. The HMS system based on NIR regulating osteoblasts with HMS responsive slow-release drugs still needs to be improved. Similarly, many studies have also utilised ultrasound to promote bone regeneration by controlling drug release from HMS, and low-frequency ultrasound may also promote osteogenesis through the BMP signalling pathway and activate the downstream pathway of SMAD1.[60, 61] However, the mechanisms underlying these actions need to be investigated further. In addition, HMS can also promote osteogenesis by improving the bone regeneration environment through electroconduction.[167] Electrical stimulation affects the proliferation and differentiation of BMSCs by regulating intracellular calcium ion uptake and ion channel activity.[58]

Hydrogel microsphere system combined with chemical cues promote bone regeneration

The bone matrix microenvironment is a complex and

sophisticated ecosystem. In addition to physical stimuli and various types of cytokine crosstalk, the bone matrix chemical environment includes ROS, oxygen, and suitable pH. These elements play a crucial role in maintaining the health and regeneration of skeletal tissues. Elevated levels of oxidative stress and ROS are one of the important factors affecting bone regeneration. HMS can be engineered to release antioxidants or other bioactive agents to reduce oxidative stress and ROS levels within the damaged area. This helps to reduce cell death, promote cell proliferation and differentiation, and ultimately facilitate bone regeneration. Yang et al.[189] modulated redox homeostasis in BMSCs and promoted refractory bone healing by constructing fullerol-hydrogel microfluidic spheres *in situ*. Fullerol has antioxidant properties that can directly react with ROS and neutralise their activities. In addition, it enhances the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, while inhibiting nicotinamide adenine dinucleotide phosphate oxidase activity. By reducing nicotinamide adenine dinucleotide phosphate oxidase-mediated ROS production, fullerol maintains the stability of the mitochondrial membrane potential and reduces electron leakage and ROS production, resulting in an intra- and extracellular antioxidant effect (**Figure 10C**). Oxygen concentration is one of the important factors affecting the growth and differentiation of osteoblasts. HMS can also be designed as a carrier capable of regulating the local oxygen concentration, making it suitable for osteoblast growth and directed differentiation. Seyyed Nasrollah et al.[88] thermally designed and prepared MgO_2-loaded PLGA microspheres to improve the oxygen and pH environment in the bone regeneration microenvironment.

Summary and Future Directions

This review article focuses on the specific content of the bone regeneration microenvironment and the related mechanism by which HMS regulates this microenvironment to achieve bone regeneration. We have discussed in detail the preparation materials and methods and the functional characteristics of HMS, listed the application scenarios of the HMS system in bone tissue engineering, and elaborated on the relevant mechanisms by which HMS promotes bone regeneration by regulating key elements in the bone regeneration microenvironment. Overall, the main advantages of using the HMS system to promote bone regeneration include: 1) High loading efficiency: HMS has high porosity and large specific surface area, providing more sites for drug/stem cells to be delivered; 2) Controlled release: by adjusting the physicochemical properties of HMS, different release modes are selected according to different pathological microenvironments to achieve more accurate spatiotemporal release. 3) Versatility: HMS can be easily modified and specially designed to adapt to and regulate the physiological, chemical, and physical microenvironment in bone regeneration; and 4) Strong drug/stem cell protective ability: HMS can encapsulate drugs/stem cells into microspheres, reduce external forces and stimuli of harmful environments during delivery, and protect drug/stem cell activity. In conclusion, microspheres have high loading efficiency, controllable release, versatility, and drug/stem cell protective properties that can help regulate the microenvironment of bone regeneration and promote the repair and regeneration of bone tissue. In addition, there are challenges with respect to the clinical application of HMS: 1) Biocompatibility and Cytotoxicity: As an *in vitro* substance, HMS must guarantee the biocompatibility of all its components. It is essential to conduct a comprehensive evaluation of the biocompatibility and potential cytotoxicity of certain HMS-loaded drugs, particularly metal nanoparticles, prior to commencing clinical trials. 2) Controlled release: The porous structure and customisable physicochemical properties of HMS provide adjustable controlled release capabilities. Consequently, the drug-carrying HMS system must ensure that the necessary controlled release properties for therapeutic efficacy are achieved upon entry into the organism, thereby avoiding potential therapy-related side effects associated with sudden release or lack of release. 3) Biodegradability: As implants, the timely degradation of HMS from the body is as important as their stable presence during treatment. The components of the HMS and their *in vivo* degradation products must not adversely affect the target tissue (e.g. induction of inflammation, infection).

In recent years, various preparation technologies of the HMS system have made great progress. These technologies can prepare a multifunctional HMS system for bone tissue engineering. In view of the complexity of the bone regeneration microenvironment and the need for a comprehensive regulation strategy, future HMS system designs and research based on the physiological, chemical, and physical microenvironment of bone regeneration microenvironment should be improved in the following ways:

1. In the physiological microenvironment of bone regeneration, nerve-blood-vessel-immune-bone crosstalk is a relatively complex network.[156, 175] Various functional components can accurately deliver through HMS to achieve the functional crosstalk and regulation of nerve, blood vessels, immune system, and osteocytes in the physiological microenvironment of bone regeneration and promote bone regeneration.

2. The crosstalk between the physiological and chemical microenvironments is an important factor in bone regeneration. For example, neurocytes can produce CGRP, promote macrophage polarisation towards M2 type,[190] and downregulate the expression of ROS-related genes in inflammatory cells,[191, 192] remodelling the local immune balance. Therefore, by constructing HMS with the comprehensive regulation function of the physiological and chemical microenvironment, the efficacy of synergistically promoting bone regeneration can be achieved.

3. In pathological conditions such as open fractures and osteoporosis, the osteogenic capacity of bone regeneration-associated cells (e.g. BMSCs, endothelial cells, neurocytes) significantly decreases, directly disrupting the physiological microenvironment necessary for bone regeneration. Concurrently, the dysfunction of immune cells (e.g. neutrophils, macrophages) in bone lesions can lead to elevated ROS levels and low pH, further compromising the local chemical microenvironment and hindering bone regeneration. Beneficial physical stimulations have been proven effective in regulating the

functions of various functional and immune cells. For instance, the physical signals transduced by the HMS system—such as electrical stimulation, mechanical force, and light stimulation—promote osteogenic differentiation, angiogenesis, and neurocyte regeneration, while regulating the functions of inflammatory cells like macrophages and neutrophils, thereby remodelling both the physiological and chemical microenvironments.[193-197] Therefore, further development of the HMS system, capable of transducing precise physical signals, may enable the accurate regulation of various functional and immune cells. This would remodel the physiological and chemical microenvironments necessary for bone regeneration, accelerating fracture healing and bone defect repair. Moreover, it holds the potential to make significant progress in the treatment of chronic bone diseases such as osteoporosis.

4. In the bone regeneration microenvironment, physiological, chemical, and physical microenvironments work jointly to regulate bone regeneration. Based on the existing single-factor or double-factor regulation, realising the comprehensive regulation of the HMS system on the bone regeneration microenvironment can enable the precise and intelligent regulation of bone regeneration and accelerate this regeneration and repair of bone defects.

5. Currently, few studies have focused on the use of HMS to treat diseases such as osteoporosis and bone tumours. Combining HMS with emerging nanotechnology and gene therapy could lead to considerable breakthroughs in these areas.

Limitations

Limitations of literature coverage

Review articles exploring the application of HMS in bone regeneration to modulate the regenerative microenvironment may be limited by the scope of literature coverage. Owing to the selection of research literature and search strategies, certain key studies may have been missed, especially in relatively new or niche research areas. This limitation in coverage may result in the review providing a less-than-comprehensive description of current technologies and research advances.

Updatability issues

Owing to the rapid advancement of HMS in the field of bone regeneration, the review articles may not have been written in a way that covers the latest research findings and technological advances. This time lag may affect the timeliness of the review and its accurate reflection of the current state of the field.

Lack of data on the use of HMS for clinical conversion

The data on the specific clinical effects of HMS in bone regeneration in the current study may be insufficient or incomplete, which may prevent the review from fully assessing the practical value of the technology and the future direction of its development.

Author contributions

Conceptualization: PZ, QQ; investigation: PZ, QQ, XC, HX; writing - original draft: PZ, QQ, XC; software: XC, HX, DF; methodology: HX, DF, DW; supervision: DF, YL; manuscript review & editing: DW, YL;: supervision, and project administration: YL. All authors read and approved the final version of the manuscript.

Financial support

This work is supported by the National Natural Science Foundation of China (Nos. 82102578, 82472404), Special Project for the Central Government to Guide the Development of Local Science and Technology in Sichuan Province (No. 2023ZYD0071), China Postdoctoral Science Foundation (No. 2022M720603), National Natural Science Foundation of Sichuan (No. 2024NSFSC0678), Natural Science Foundation of Chongqing (No. CSTB2022NSCQ-MSX0104), Research Project of Health Commission of Sichuan Province (No. 2023-1601), Research Project of Nanchong Science and Technology Bureau (Nos. 22SXJCQN0004, 22SXQT0308), and Research Project of the Affiliated Hospital of North Sichuan Medical College (Nos. 2023ZD002, 2023-2ZD001).

Acknowledgement

None.

Conflicts of interests statement

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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Received: June 27, 2024
Revised: August 12, 2024
Accepted: September 13, 2024
Available online: September 28, 2024