From the microspheres to scaffolds: advances in polymer microsphere scaffolds for bone regeneration applications

Shuhao Yang[1,2,#], Haoming Wu[5,#], Chao Peng[3,#], Jian He[6], Zhengguang Pu[3], Zhidong Lin[7], Jun Wang[1,2], Yingkun Hu[1,2], Qiao Su[8], Bingnan Zhou[9], Xin Yong[10], Hai Lan[3,], Ning Hu[1,2,*], Xulin Hu[3,4,*]*

Key Words:
biomimetic scaffolds; bone tissue engineering; polymer microspheres; regenerative medicine

From the Contents

ABSTRACT

The treatment and repair of bone tissue damage and loss due to infection, tumours, and trauma are major challenges in clinical practice. Artificial bone scaffolds offer a safer, simpler, and more feasible alternative to bone transplantation, serving to fill bone defects and promote bone tissue regeneration. Ideally, these scaffolds should possess osteoconductive, osteoinductive, and osseointegrative properties. However, the current first-generation implants, represented by titanium alloys, have shown poor bone-implant integration performance and cannot meet the requirements for bone tissue repair. This has led to increased research on second and third generation artificial bone scaffolds, which focus on loading bioactive molecules and cells. Polymer microspheres, known for their high specific surface areas at the micro- and nanoscale, exhibit excellent cell and drug delivery behaviours. Additionally, with their unique rigid structure, microsphere scaffolds can be constructed using methods such as thermal sintering, injection, and microsphere encapsulation. These scaffolds not only ensure the excellent cell drug loading performance of microspheres but also exhibit spatial modulation behaviour, aiding in bone repair within a three-dimensional network structure. This article provides a summary and discussion of the use of polymer microsphere scaffolds for bone repair, focusing on the mechanisms of bone tissue repair and the current status of clinical bone grafts, aimed at advancing research in bone repair.

***Corresponding authors:**
Xulin Hu,
huxulin1993@163.com;
Ning Hu,
huncqjoint@yeah.net;
Hai Lan,
13714777@qq.com.

#Author equally.

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Introduction

Millions of people worldwide experience the destruction and loss of bone tissue due to infection, tumour resection, and trauma each year, including more than 2 million patients who require bone grafting, which represents a significant challenge in clinical management today.[1, 2] Under normal physiological conditions, bone tissue has a unique ability to heal itself; however, bone defects are often associated with complex pathologies (e.g., comorbidities with diabetes, genetics, and infections), and bone loss beyond critical dimensions increases the risk of osteochondral disjunction as well as delayed bone healing.[3] For such bone defects that are beyond the ability of bone tissue to heal on its own, bone regeneration is usually assisted by bone grafts, currently, the "gold standard" for the treatment of bone defects is still autologous or allogeneic bone grafting, which often carries with it a series of risks such as limited bone source, immune rejection and infection.[4] An artificial bone scaffold is a safer, simpler and more feasible bone graft that can fill the bone defect area while maintaining the physiologic environment of the defect area to promote bone regeneration. Ideal artificial bone scaffolds maintain three main biological properties in promoting bone regeneration: osteoconduction, osteoinduction, and osseointegration properties.[5, 6] However,

the first generation artificial scaffolds represented by titanium alloys are unable to meet today's needs for bone defect treatment due to their poor bone tissue integration and low osteoinductive properties, and second generation scaffolds loaded with active molecules and third generation scaffolds combined with cell-carrying therapies have become a popular direction of research for new artificial bone repair scaffolds.[7, 8]

Microspheres, as a type of micro- and nanoscale materials with their own high specific surface area, are capable of good cell-loading and drug-controlled release behaviours, and have been used in various fields of biotherapeutics.[9] In addition, microspheres can be assembled into tissue-engineered scaffolds with a three-dimensional (3D) porous structure either individually or in combination due to their unique rigid shape structure, which are mainly classified into microsphere-based scaffolds and incorporated microsphere scaffolds. Microsphere-based scaffolds are divided into injectable microsphere-based scaffolds and sintered microsphere-based scaffolds by the preparation method.[10, 11] Sintered microsphere-based scaffolds, which are mainly assembled with solid microspheres, can be made to achieve the mechanical strength of cancellous bone by the methods of heating to the glass transition temperature and solvent bonding.[12] Injectable microsphere scaffolds encapsulating microspheres in hydrogel not only utilise the excellent hydrophilicity and biocompatibility of hydrogel, but also its similarity to natural extracellular matrix (ECM) and injectability allowing it to be filled into the local tissues for therapeutic purposes as well as controlled release of drugs for regenerative in situ repair of bone tissue.[13] Incorporated microsphere scaffolds cleverly combine the advantages of microspheres and other types of scaffolds, which can be constructed by electrostatic adsorption and surface adhesion with existing scaffolds using microspheres as drug carriers and cell transporters, and can also use microspheres to form a network of holes inside the scaffolds to promote scaffold resorption as well as direct cell proliferation and differentiation.[10]

In addition to the ability to design customised bone repair microsphere scaffolds through different scaffold construction methods, the microsphere monomers themselves are prepared by multi-conditional preparation methods such as microfluidics, emulsion and spray drying to have porous, core-shell, and Janus structures, which greatly improve the delivery of drugs as well as active molecules and the binding efficiency of cells.[14-16] In addition, there is a huge difference between the physicochemical properties of microspheres from different substrates, and polymers have been chosen as the preferred choice for the preparation of microspheres due to their excellent degradation behaviour in the organism as well as their significant non-cytotoxicity. Natural polymers represented by gelatin, alginate and chitin have functional groups that are favourable for cell adhesion and proliferation, in addition to their hydrophilic structure that can form hydrogel-crosslinked network structures to be prepared as hydrogel microspheres, and their spherical structure with excellent physical rollability to enhance the filling in irregular tissues.[17, 18] Synthetic polymers represented by poly(lactic acid) (PLA), poly(glycolic acid) and poly(ethylene glycol) (PEG), which have the characteristics of toughness and stability that natural polymers do not have, are the most ideal materials for bone scaffolds, and poly(lactic-co-glycolic acid) (PLGA) and PLA microspheres, and as a new type of drug delivery carriers, are commercially available, which have a great prospect for application.[19-21]

In summary, in this article, we start from the mechanism of bone regeneration and describe the anatomical structure of bone tissue as well as osteoblasts and various regulatory factors involved in the process of bone regeneration, which provides readers with a better theoretical foundation. We then analyse the bone regeneration grafts currently available in the clinic, presenting the ideal needs of bone regeneration grafts and the advantages of degradable polymer microspheres in bone regeneration. For bone regeneration microsphere scaffolds, we summarised their characteristics and classification, and proposed some design strategies and performance requirements, aiming to provide some references for the construction of bone regeneration microsphere scaffolds (**Figure 1**). Finally, we summarised the preparation process of different types of microsphere scaffolds and their applications in bone tissue regeneration and put forward the prospect for the future. We believe that shortly, through multidisciplinary cooperation, we can prepare bone regenerative microsphere scaffolds with ideal performance to help bone tissue regeneration in the future.

Retrieval Strategy

The literature database for this manuscript was searched by the first author. The search was limited to high quality English language literature published between January 1990 and June 2024. Literature published in the last 5 years (June 2019 to June 2024) was predominantly used, with approximately 80% of articles published in the last 5 years and approximately 20% of articles published before 5 years. Search databases included PubMed, Web of Science, Scopus, etc. The search terms included polymer microspheres, biomimetic scaffolds, bone tissue engineering, regenerative medicine, and so on. The selection criteria included high-level English literature related to microsphere preparation, high-level English literature related to bone regeneration mechanism, and high-level English

1 Department of Orthopaedics, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China; 2 Orthopedic Laboratory of Chongqing Medical University, Chongqing, China; 3 Clinical Medical College and Affiliated Hospital of Chengdu University, Chengdu University, Chengdu, Sichuan Province, China; 4 Department of Biotherapy, Cancer Center and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan Province, China; 5 School of Preclinical Medicine of Chengdu University, Chengdu University, Chengdu, Sichuan Province, China; 6 College of Basic Medicine and Forensic Medicine, Henan University of Science and Technology, Luoyang, Henan Province, China; 7 The Second Affiliated Hospital of Chinese Medicine, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong Province, China; 8 West China School of Stomatology, Sichuan University, Chengdu, Sichuan Province, China; 9 School of Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA; 10 Key Laboratory of Birth Defects and Related Diseases of Women and Children, Department of Paediatrics, West China Second University Hospital, State Key Laboratory of Biotherapy, Sichuan University, Chengdu, Sichuan Province, China

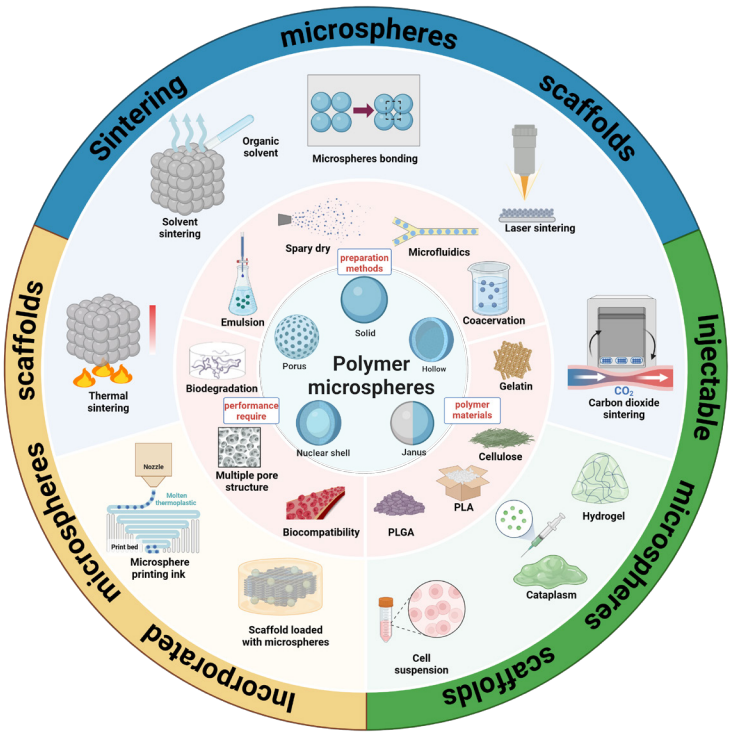

Figure 1. Construction of different types of bone repair scaffolds by screening the preparation method, materials and performance requirements of polymer microspheres. Different types of polymer microspheres can be obtained by different materials and preparation methods, mainly including solid microspheres, porous microspheres, core-shell microspheres, Janus microspheres and hollow microspheres, and polymer microspheres show good degradation behaviour, porous structure and good biocompatibility. Finally, the polymer microspheres were assembled into sintered, injectable, and incorporated microsphere scaffolds to exhibit different properties. Created with BioRender.com. PLA: poly(lactic acid); PLGA: poly(lactic-co-glycolic acid).

literature related to microspheres for bone regeneration. Most of the included literature had reliable data, and rigorous logic, and was recognised by other researchers, and at the same time, we preferred recently published literature in the same field related to the topic.

Physiology of Bone Regeneration

Bone regeneration is often accompanied by complex mechanisms in the body. At the site of bone tissue destruction, skeletal stem cells are activated and begin to divide, and these dividing cells differentiate into osteoblasts and chondrocytes to rebuild bone tissue.[22] Osteoblasts and chondrocytes gradually deposit bone tissue by secreting bone matrix proteins, leading to the gradual replacement of fibrous bone and cartilage with mature bone. Angiogenesis is the key link in the process of bone regeneration, and the new blood vessels will provide nutrients and oxygen to the bone tissue, and promote the proliferation and differentiation of osteoblasts.[23] Therefore, the mechanism of bone regeneration is a complex process, and this chapter will describe the biological process of bone regeneration in terms of the structure of bone tissue as well as the basic cells and molecules (**Figure 2**).

Anatomy of bone tissue

Anatomy of bone

The skeleton is the metabolically active connective tissue that provides levers for muscles, protects vital structures, stores minerals and growth factors, regulates mineral and acid-base balance, and offers structural support for haematopoietic function and facilitates movement.[24] The two main structural types of bone are cortical bone and cancellous bone.[25] Cortical bone, the outer layer of the skeleton, is dense and solid, featuring periosteal and endosteal surfaces. Periosteal activity is important for postural growth and fracture repair. Bone formation typically exceeds bone resorption at the periosteal surface, thus the diameter of bones generally increases with age. The endosteum has a higher remodelling activity than the periosteum; bone resorption often exceeds bone formation at the endosteum, thus the marrow space usually enlarges with age. In the human body, cortical bone is extensively present in long bones (such as the humerus and tibia), flat bones (such as the skull and sternum), and short bones (such as the carpal bones).[24] Cortical osteons are called Haversian systems. Haversian canals, derived from osteoblasts, are narrow channels connecting the trabeculae and permeate the entire bone tissue.[26] They are primarily located in the shaft parts of bones, such as the central axis of long bones. Haversian canals, composed of blood vessels and nerve tissues, serve to transport nutrients and oxygen and to transmit nerve signals.[27] They are connected to the entire skeletal system through endosteal congestion, and bone marrow, providing necessary nutrients and support for physiological activities.[28]

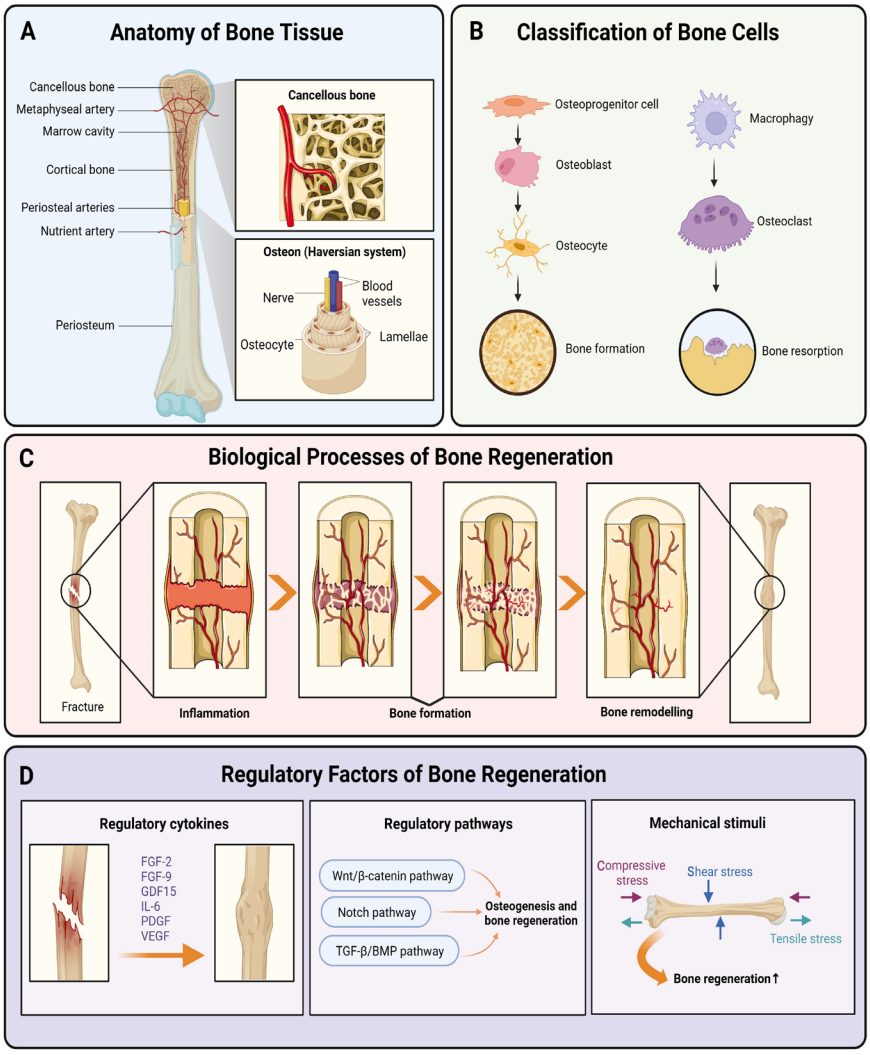

Figure 2. Coordination of various cells and molecules during bone regeneration. (A) Bones are composed of cortical bone and cancellous bone. Cortical osteons are called Haversian systems, with the Haversian canals in the center, containing blood vessels and nerves. (B) The cellular components of bone tissue are osteoprogenitor cells, osteoblasts, osteocytes, and osteoclasts. (C) Bone regeneration process can be divided into three stages: inflammation, bone formation, and bone remodelling. (D) Bone regeneration is governed by an intricate network of regulatory cytokines and signalling pathways including FGFs, GDF15, IL-6, PDGF, VEGF, Wnt/β-catenin signalling pathway, Notch signalling pathway and BMPs signalling pathway. Mechanical stimulation can also promote bone regeneration through the transmission of mechanical forces and the activation of cell signalling pathways. Created with BioRender.com. BMP: bone morphogenetic protein; FGFs: fibroblast growth factors; GDF15: growth differentiation factor 15; IL-6: interleukin 6; PDGF: platelet-derived growth factor; VEGF: vascular endothelial growth factor.

Cancellous bone, located within the cortical bone, constitutes the inner structure of the skeleton and is relatively lighter. The porosity of trabecular bone ranges from 50% to 90% and is filled with bone marrow. Cancellous bone plays important roles in the skeleton, including providing strength and support to bones, storing bone marrow, participating in the metabolism of calcium ions, and processes of skeletal remodelling.[25] Cancellous bone also exhibits higher metabolic activity and remodelling rates and responds more quickly to mechanical stimuli because primary bone cells are located on the surface, closer to circulating growth factors and cytokines.[24] Cancellous bone is composed of plates and rods of trabeculae.[25] These trabeculae are net-like within the bone. They are distributed in a uniform direction, forming a complex 3D lattice structure. The presence of trabeculae increases the strength of the bone, allowing it to withstand loads from bodily movement and external pressure. Cancellous bone has a richer cell and vascular supply and higher metabolic activity than cortical bone.[29] Through the dense arrangement and interconnection of these structures, the human skeletal system is capable of providing support and protection, while also participating in the metabolism and growth processes of bones.

Anatomy of blood vessels

Bone is a highly vascularised connective tissue. The vasculature of bone plays a key role in the processes of bone development,

regeneration, and remodelling.[30] Vessels, primarily comprising arteries, veins, and capillaries, not only provide the skeletal system with oxygen and nutrients and clear metabolic waste from bones, but also deliver specific hormones, growth factors, and neurotransmitters secreted by other tissues, maintaining the viability of bone cells and stimulating their activity.[31] The bone marrow cavity is replete with abundant bone marrow, including yellow and red marrow; the cortical bone contains numerous small blood vessels that penetrate it to supply nutrients and oxygen to the bones. The ends of long bones are composed of epiphyses, which contain a rich vascular network, providing essential nutrients for the growth and development of the epiphyses. Between the trabeculae there is a series of small vascular branches, ensuring blood supply to the whole skeletal system. The periosteum, rich in blood vessels and nerves, also plays a significant role in the healing of fractures, bone growth, and repair processes.[32]

Regulatory factors and bone cells of bone regeneration

The cellular components of bone tissue include osteoprogenitor cells, osteoblasts, osteocytes, and osteoclasts. Osteocytes are the only cells residing within the bone matrix, while the other three types are located at the periphery of the bone tissue.[33] Osteoprogenitor cells are the stem cells in bone tissue that, during periods of bone growth and development or bone remodelling and repair in adulthood, can proliferate and differentiate into osteoblasts.[34] Osteoblasts, primarily located within the bone tissue and usually encapsulated in the bone matrix, are multinucleated cells that can promote the formation of new bone matrix by secreting collagen, osteocalcin, and other components.[24] Moreover, they can communicate and collaborate with bone-resorbing cells to balance bone resorption and formation, thus maintaining skeletal health.[35] As polarised cells, osteoblasts secrete osteoid into the bone matrix.[34] Osteoblasts originate from mesenchymal stem cells (MSCs), which can differentiate into various cell types, including chondrocytes, myocytes, osteoblasts, and adipocytes.[36] MSCs are capable of self-renewal, continually producing new stem cells to maintain their multipotency. Furthermore, they can regulate immune responses, alleviate inflammation, and promote tissue repair.[37] Osteocytes are distributed within the bone matrix and represent terminally differentiated cells. Their osteogenic activity is significantly lower than that of osteoblasts, yet they comprise over 90% of the cells in adult bone. Although less active compared to osteoblasts, osteocytes play a central role in determining and maintaining bone structure.[38] Apoptosis of osteocytes has been recognised as a chemotactic signal for osteoclast-mediated bone resorption. Studies have shown that apoptotic osteocytes are phagocytosed by osteoclasts during the process of bone resorption.[39] Osteoclasts originate from the mononuclear phagocyte system in the bone marrow and become mature through a series of differentiation and activation processes.[40] When bone tissue needs remodelling or repair, osteoclasts adhere to the bone surface and commence resorption by secreting acid proteases that dissolve the mineral and collagen in bone tissue, thus facilitating bone remodelling and regeneration.[40] Ligand to receptor activator of nuclear factor-κB and macrophage colony-stimulating factor are two cytokines essential for osteoclast formation, mainly produced by osteoblastic and bone marrow stromal cells in both membrane-bound and soluble forms.[41, 42]

Bone regeneration is influenced by a multitude of regulatory cytokines and signalling pathways. Key cytokines have been identified as playing a critical role in modulating the proliferation, differentiation, and osteogenic capacities of bone cells. Members of the fibroblast growth factors (FGFs) family, including FGF-2 and FGF-9, have been shown to stimulate the proliferation, differentiation, and survival of bone cells, and play an important role in fracture healing and bone regeneration.[43, 44] Growth differentiation factor 15 is considered to play a significant role in repairing fracture and bone injury, enhancing the proliferation and osteogenic capacity of bone cells, and regulating bone tissue regeneration in conjunction with other cytokines.[45] Interleukins play an essential role in the immune system, they also regulate the functions of bone cells; for example, interleukin 6 can stimulate the proliferation and differentiation of bone cells.[46] Additionally, other cytokines, such as transforming growth factor β (TGF-β), platelet-derived growth factor, and vascular endothelial growth factor, are also widely applied in bone tissue engineering and bone regeneration research.[47-49]

The Wnt/β-catenin signalling pathway plays an important role in bone regeneration, promoting the proliferation and differentiation of bone progenitor cells and regulating the functions of osteoblasts through the activation of intracellular β-catenin protein.[50] During the early stages of bone healing, inhibition or activation of the Wnt pathway can suppress the differentiation of MSCs into osteoblasts. In the later stages, as undifferentiated cells transition to the osteoblast lineage, the Wnt pathway positively regulates osteoblasts.[51] The Notch signalling pathway is a highly conserved ligand-receptor signalling system that plays an important role in cell proliferation, differentiation and development.[52] Studies demonstrate that the Notch-2 signalling pathway, particularly the Jag-1 signalling, is involved in the differentiation of MSCs into osteoblasts.[53] Additionally, it has been demonstrated that the binding of Jag-1 to biomaterials can facilitate the differentiation of osteoblasts.[54] Bone morphogenetic proteins (BMPs) are a significant group of growth factors regulating bone regeneration. Intracellularly, BMPs activate signalling pathways by binding to their receptors on the cell membrane, promoting the proliferation and differentiation of bone progenitor cells, thus fostering the regeneration of bone tissue.[55] TGF-β is a multifunctional regulator playing a pivotal role in the proliferation and differentiation of bone cells, as well as in the synthesis of bone matrix. Moreover, important pathways such as phosphoinositide 3-kinase/protein kinase B (Akt)/mammalian target of rapamycin, mitogen-activated protein kinase, platelet-derived growth factor, insulin-like growth factor, FGF, and Ca^{2+} signalling pathways also play critical roles in bone regeneration, with these pathways interacting with one another, collectively modulating the process of bone regeneration.[56-61]

Mechanical stimulation can promote bone regeneration through the transmission of mechanical forces and the activation of cell

signalling pathways.[62] Mechanical stimulation can have a direct effect on osteoblasts, thereby affecting their gene expression, protein synthesis, and proliferation and differentiation. For instance, tensile and compressive forces promote the proliferation of bone cells and the synthesis of bone matrix, while shear stress is beneficial to the differentiation of osteoblasts.[63] Furthermore, mechanical stimulation can also regulate the function of bone cells. By simulating normal physiological loads, such as exercise and weight-bearing, mechanical stimulation can enhance the functional properties of bone cells, such as mechanical stability and fatigue resistance.[64]

Biological processes of bone regeneration

The bone regeneration process encompasses three partially overlapping stages: inflammation, bone formation, and bone remodelling.[65] In the inflammatory phase of bone regeneration, trauma or fracture leads to tissue injury, activating an inflammatory response of the immune system. Fracture disrupts blood vessels, and causes hemorrhage which triggers blood clot formation at the wound site, and then forms a thrombus to prevent further blood loss. Inflammatory cells such as neutrophils and monocytes are rapidly recruited from surrounding tissues and circulation to the wound site, guided by chemotactic factors. These cells migrate toward the area of injury, releasing a plethora of inflammatory mediators, including cytokines, chemokines, and growth factors, at the fracture locus. Such mediators attract additional inflammatory cells and promote angiogenesis. Inflammatory cells also clear the wound area by phagocytising and degrading dead cells and thrombi.[66, 67] During the regenerative stage, stem cells and progenitor cells commence division and proliferation, differentiating into collagen-producing cells and chondrocytes, eventually transitioning into osteoblasts. These cells produce the bone matrix and ultimately form the initial bone tissue, a process known as chondrogenesis or osteogenesis. The nascent bone tissue gradually matures and fills the injured site.[67, 68] Bone remodelling is a very complex process involving osteoclast-triggered bone resorption, a transitional stage from resorption to new bone formation. This orchestrated activity among osteoclasts, osteoblasts, and osteocytes results in the replacement of old bone with new bone.[69, 70] Together they comprise a temporary anatomical structure called the basic multicellular unit.[71-73] Proper bone remodelling is vital for promoting speedy fracture healing, facilitating skeletal adjustment to mechanical strain, and maintaining optimal calcium balance.[74]

Bone Regeneration Grafts

Although bone tissue regeneration is possible through specific cytokines and signalling pathways, the destruction of bone tissue is often accompanied by complex peripheral damage, including critical size bone defects, infected bone defects, diabetes mellitus, genetics, and poor lifestyle (smoking and alcoholism) that greatly increase the healing time for bone regeneration and result in bone nonunion.[75] At the same time, the imbalance in the surrounding immune microenvironment also leads to the destruction of peripheral neovascularisation as well as the migration of normal osteoblasts which further slows down bone healing. Therefore, effective bone grafts or substitutes are needed to provide a good osteogenic platform for bone regeneration while preventing further loss of bone tissue.[76]

Mechanisms of bone grafts for bone regeneration

Ideal bone grafts should have the three main characteristics of osteoinduction, osteoconduction and osteogenic capacity thus fully integrating the graft with the bone tissue.[77, 78] In the case of osteoinduction, MSCs are recruited on the graft platform and then differentiate into various cell types, which are often regulated by growth factors, hormones and proteins. Finally, good osteogenesis requires the ability of the graft to synthesise new bone tissue, which promotes the proliferation of MSCs, osteoblasts and osteocytes.[79, 80]

Both fresh autologous grafts and exogenous grafts in the host body first form a large amount of inflammatory factors during the haematoma period, which is also accompanied by a high release of cytokines and growth factors thus effectively recruiting MSCs and macrophages. Subsequently, fibrotic vascular tissue develops during the inflammatory process.[81] The cancellous bone, as the largest and most metabolically active part of the bone tissue, is the primary site of bone tissue repair, and the grafts filling the cancellous bone sites produce osteoblast-like cells by recruiting MSCs and differentiating into osteoblasts. After a minimum of 6 months of metabolic processes, the osteoid is mineralised to form new bone. In contrast to cancellous bone, cortical bone regeneration is often mediated by osteoclasts and accompanied by several years of evolution.[77]

Current status of clinical bone grafting

Currently, the "gold standard" for the treatment of damaged or non-healing bone tissue is still autologous or allogeneic bone grafting, but this is also limited by the shortcomings of limited bone volume, immune rejection, and high price.[82] Therefore, more long-term, effective, and safe grafts need to be designed and researched, which are mainly divided into the first generation of scaffolds: the use of synthetic scaffolds in a stand-alone fashion; Second generation scaffolds: scaffolds loaded with active molecules; third generation: scaffolds incorporating cell-loaded therapies. Currently, the first generation of stents has been widely put into phase I, II or III clinical trials, and some are even widely used in the clinic.[83] Titanium implants, as the first metallic implants, have significant mechanical strength and can be 3D printed to correspond to the patient's anatomy through structural parameterisation (NCT03292679, NCT03057223, and NCT03242330). However, as first-generation bone grafts, they are predominantly inert materials and are prone to non-specific immune reactions and aseptic loosening of the tissue. Such metallic implants degrade as bone tissue over time due to stress shielding. Second-generation bone grafts are coated and modified with biodegradable biomaterials based on the first-generation but have never been able to heal bone quickly from the combined action of osteoblasts, ECM, and biochemical signalling. In addition, we have collected the mainstream clinical bone implant products and summarised their main components and application specifications (**Table 1**).

Table 1. Bone grafts for clinical research

Material	Performance parameter	Application form	Product name	Manufacturer	FDA 510(k) number
10% porcine type I collagen, 90% bovine bone mineral	Porosity: 70–75%, pore size: 300–1500 μm	Chunk	Bio-Oss Collagen	Geistlich, Wolhusen, Switzerland	K122894
Bovine type I collagen, hydroxyapatite	Porosity: 70–88%, pore size: 50–500 μm	Chunks, granules, cylinders	Bongold	Allgens, Beijing, China	K141725
Bovine collagen, pork bone minerals	Porosity: 73.42–77.26%, pore size: 0.003–360.86 μm	Cylinders	DSM Biomedical Dental Bone Graft	DSM Biomedical, Exton, PA, USA	K193212
Bovine collagen, calcium salts, phosphate	Porosity: 93%, pore size: N/A	Spongy, doughy	CopiOs	Zimmmer, FL, USA	K033679
70% bovine I collagen, hydroxyapatite	Porosity: 95%, pore size: 4–200 μm	Stripe	HEALOS	Johnson & Johnson, NJ, USA	K012751
Bovine collagen, pork bone minerals	Porosity: N/A, pore size: N/A	Chunks	LegoGraft	Purgo Biologics, Gyeonggi-do, Korea	N/A
Bovine type I collagen, biphasic bioceramics (15% hydroxyapatite and 85% β-tricalcium phosphate)	Porosity: 89%, pore size: N/A	Stripe, doughy	MASTERGRAFT Strip/Putty	Medtronic, Minneapolis, MN, USA	K082166, K081784
Bovine type I collagen, biphasic bioceramics (60% hydroxyapatite and 40% β-tricalcium phosphate)	Porosity: > 90%, pore size: N/A	Stripe	MCS Bone Graft	Bioventus, Durham, NC, USA	K162860
8% type I collagen, 92% minerals (30% hydroxyapatite and 70% tricalcium β-phosphate)	Porosity: 70%, pore size: 500–1000 μm	Cylinders	OSTEON III	Dentium, Suwon-si, Korea	K153676
45% bovine type I collagen, 55% synthetic calcium phosphate	Porosity: N/A, pore size: N/A	Stripe, chunks, doughy	OssiMend	Collagen Matrix, Paramus, NJ, USA	K052812
PMMA bone cement	Porosity: N/A, pore size: N/A	Injectable	Spineplex	Stryker, Portage, MI, USA	K151125, K162062
Calcium sulfate, 4% tobramycin sulfate, stearic acid	Porosity: N/A, pore size: N/A	Injectable	Osteoset	Wright Medical Technology, Memphis, TN, USA	K150841

Note: N/A: not applicable.

In conclusion, many bone implant products currently used in clinical practice consist of inorganic salts combined with collagen. While this composition creates a beneficial environment for bone formation, it also demonstrates drawbacks such as irregular degradation, limited adaptability, and high cost. Therefore, there is potential to enhance the incorporation of bioactive factors and cells in existing clinical products, as well as explore the use of other alternative materials for scaffold development.

Microsphere Preparation for Bone Regeneration Scaffolds

Ideal bone repair scaffolds aim to bionically mimic natural bone in multiple dimensions, most notably by reconstructing bone tissue hierarchies at the nano- to macroscale.[84] Conventional techniques (e.g., freeze casting, phase separation, electrostatic spinning) often fail to satisfy the homogeneous regulation of scaffold size pore diameter. At the same time, 3D printing may likewise receive the limitation of low print resolution to prepare supercritical-size bone defect filling scaffolds. Micro- and nanostructured microspheres can be used to prepare biomimetic scaffolds conforming to macroscopic/microscopic multilevel structures by utilising stacking bonding methods and inclusion injection through their unique structures, and the microsphere materials and methods for constructing such scaffolds will be described in detail in this chapter.

Microsphere design for bone regeneration scaffolds

In recent years, the physicochemical properties of tissue-engineered scaffolds as well as the modulation of cell adhesion, proliferation and differentiation behaviours can be influenced by microscale modulation, and at the same time, the microstructures can mimic the trabecular structure of natural bone tissues to further improve the osseointegration properties of the grafts. Polymer microspheres, as a representative of micron-sized materials, have been widely used in various fields of medicine due to their unique controlled-release ability, which has long been used for drug delivery, as well as their high specific surface-area ratio that allows for better integration with the surrounding interface.[85] In addition, microspheres, as independent tiny units capable of combining with bioactive factors and stem cells to achieve gradient release in spatiotemporal sequences, and complex structures such as porous microspheres, core-shell microspheres, and

Janus microspheres can be obtained by various preparative means, making them an ideal tool for the design of multilevel structures and the integration of bio-interfaces.[86]

Polymer microspheres prepared from degradable and non-degradable materials have been investigated for different applications. However, nonbiodegradable polymers cannot be effectively removed after implantation. Additionally, microspheres' controlled drug release rates cannot be achieved using these polymers. Compared with non-degradable biomedical material microspheres, degradable microspheres exhibit many advantages, which are related to (a) good biocompatibility, (b) biodegradability, (c) controllable product quality and repeatability, and (d) the rate of polymer degradation can be adjusted by changing the molar ratio or viscosity.[87-89] Multifunctional microspheres prepared based on degradable materials have inherent properties, making them very suitable as controlled release carriers of drugs for the functional repair of various tissues. Depending on particle size uniformity, external morphological characteristics, and dispersibility, the application value of degradable microspheres may be limited.[90, 91] For example, the release rate of the loaded substance of microspheres requires a uniform particle size. The uniform microspheres exhibit controllable quality and good repeatability, which is beneficial to control and predict the drug release kinetics.[92] The most critical point in the design strategy of degradable microspheres is to determine the specific biomaterial composition and controlled release period.[93] Parameters such as the composition and morphology of degradable microspheres are vital for repairing specific tissues.[90] For these reasons, there is an increasing number of studies on scaffolds composed of microspheres or the inclusion of microspheres as one component within other scaffolds.

Degradable polymer microsphere materials

Degradable materials refer to materials whose chemical structure changes significantly in a short period under certain conditions or time after use, resulting in decreased physical properties and eventually absorbed by the environment. There are many kinds of biodegradable polymer microspheres synthesised at present, which are mainly divided into natural polymers and synthetic polymers. In this section, we will review the types of biodegradable materials and the polymer microspheres with different properties obtained by different preparation methods (**Figure 3**).

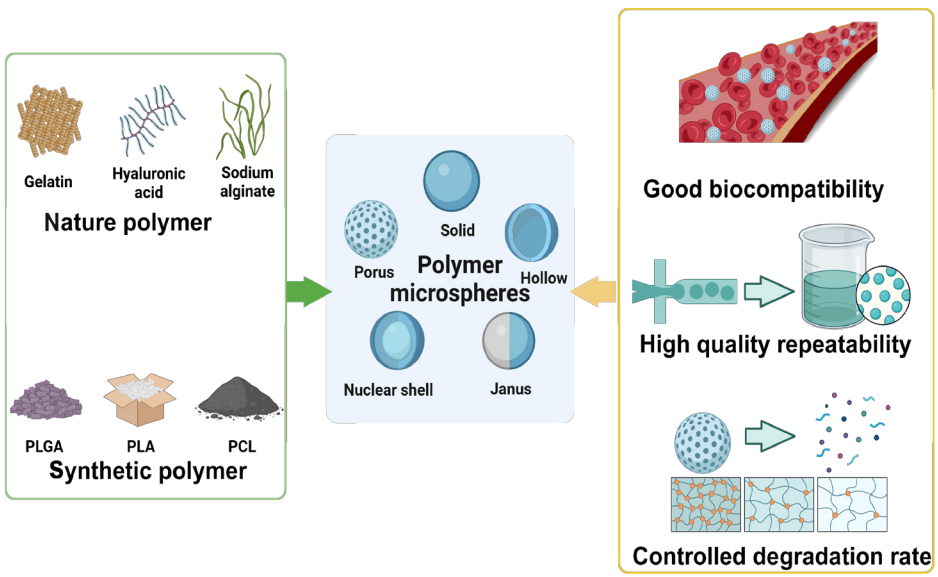

Figure 3. The performance advantages of polymer microspheres constructed from different materials. Different types of polymer microspheres can be synthesised from natural and synthetic polymers, which mainly have good biocompatibility, high repeatability and controlled degradation behaviour. Created with BioRender.com. PCL: polycaprolactone; PLA: poly(lactic acid); PLGA: poly(lactic-co-glycolic acid).

Natural polymers

Natural polymers are renewable materials with abundant sources and low prices that exist in nature.[94] Proteins and polysaccharides such as albumin, globulin, gelatin, collagen, dry enzymes, starch, cellulose, lignin, pectin, chitosan, sodium alginate, hyaluronic acid, dextran were used for biomedical, food, and cosmetic fields.[95-98] However, their insufficient mechanical properties, uncontrolled degradation, and potential immunogenicity greatly limit their application *in vivo*.

Synthetic polymers

Synthetic polymer materials are mostly aliphatic polyesters with ester groups in their molecular structures, which microorganisms or enzymes can decompose.[99, 100] The final products of safe and non-toxic polyester polymers are carbon dioxide and water, which will not remain in the body and thus will not affect the normal function of cells.[101] Among them, polycaprolactone (PCL),[102] PLA,[103] and PLGA[104] are the most widely studied as degradable materials. Moreover, more

researches focus on blended polymer materials. As a general guideline, two or more polymer compounds are usually selected for blending or copolymerisation.[105] For the reasons of regulating the degradation rate, mechanical strength, and hydrophilicity/hydrophobicity of polymers, many researchers have tried to copolymerise PCL with lactic acid and glycolic acid to obtain copolymers with a wider application range.[106, 107]

There are also synthetic polymer materials such as poly(trimethylene carbonate) (PTMC), poly(hydroxyalkanoate) and polyketals to construct drug-controlled release microspheres.[108-111] Polyanhydrides are prone to surface erosion due to the rapid hydrolysis of their anhydride bonds, while polyketides are pH-sensitive based on their pH sensitivity. Regardless of the microsphere-based strategy used to make them, all degradable microspheres, at least initially, possess more than one biodegradable component.

Preparation of degradable polymer microspheres

Degradable microspheres have various morphologies, such as spheres, microcapsules, polymer colloids, and microgels.[112] Microsphere construction methods typically include single/double emulsion solvent evapouration, spray drying, phase separation, simple and complex coacervation, and interfacial polymerisation.[113-116] The most severe application limitation of the microspheres produced by these methods is the wide size distribution. However, a specific release rate and desired route of administration generally require a specific sphere size and size distribution. This part depicts two fundamental parts: the physical and chemical methods used to create degradable microspheres (**Figure 4**).

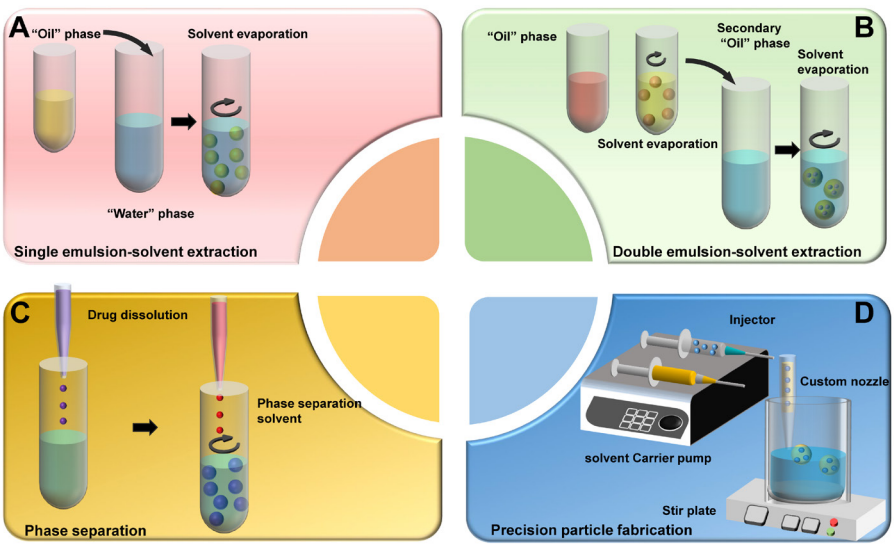

Figure 4. Different processes for the preparation of microspheres. (A) Single emulsion-solvent extraction. (B) Double emulsion-solvent extraction. The emulsion method is used to prepare microspheres by introducing hydrophilic and hydrophobic monomers into the oil-water interface system to construct the oil-water interface of water in water, and then polymerise on the interface to prepare anisotropic polymer microspheres. (C) Phase separation. The original is to add inorganic salts or non-solvent substances in the mixture of drug and polymer carrier as a coagulant to make the solubility of the polymer suddenly decrease, so that it can be separated from the mixed solution, and wrapped in the surface of the drug to form a protective layer, and then the protective layer solidified by a certain method. (D) Precision particle fabrication. The principle of microfluid-controlled preparation of microspheres is to achieve uniform mixing of materials through microchannel flow, and then form droplets or bubbles in microchannels. Then, by controlling the size and frequency of droplets or bubbles, semi-solid materials gel on the surface of droplets or bubbles, and finally, microspheres with a certain shape and size are generated through solidification and dispersion. Created with Microsoft PowerPoint 2016.

Chemical methods

The emulsification-solvent evapouration technology is a method to fabricate microspheres by removing volatile solvents in the dispersed phase from the emulsion, which is currently the most widely used method for preparing microspheres. The types of emulsions used in the preparation of microspheres can roughly be classified into the single emulsion (O/W type and O/O type)[117] and double emulsion (W/O/W type and S/O/W type)[118] methods. Simple preparation conditions and no need for specialised equipment are the most significant advantages of the emulsion-solvent evapouration method for microsphere construction.

Another widely used microsphere preparation technique is phase separation,[119] which can encapsulate drugs with different properties into the microspheres. This method is divided into a simple coacervate phase separation method and a coacervate phase separation method, which utilises the physicochemical properties of polymers.[120] However, this technology requires a large amount of organic solvent to assist compared with the emulsification-solvent evapouration method, but the

microspheres obtained have lower drug loading and larger particle size. Therefore, the choice of the microsphere preparation method depends on the encapsulated material's properties, such as lipid-water partition coefficient, molecular weight, chemical structure, and stability.

Physical methods

To protect the activity of biologically active substances, various physical approaches have been developed to fabricate degradable microspheres that can be loaded with unstable drugs. Physical methods protect biological activity more than chemical methods in terms of avoiding high-speed stirring and prolonged contact between drugs and organic solvents during the emulsification process. Physical techniques, such as spray-drying,[121] spray-freeze-drying,[122] supercritical fluids,[123] ultrasonic physicochemical methods,[124] and inkjet printing techniques,[125] have the potential to construct degradable microspheres with controllable and tunable properties.

The spray/spray freeze-drying method can effectively protect the drug's biological activity in the microspheres. However, the drug release rate is fast and accompanied by a burst effect. The particle size and yield of microspheres constructed by this method depend on the polymer concentration and pump speed. The supercritical fluid method can prepare relatively monodisperse microspheres with small particle sizes, and the preparation process is similar to the spray drying method. Moreover, spray freeze drying is suitable for a polymer with higher glass transition temperatures.

The ultrasonic atomisation method can obtain microspheres with different particle size ranges by adjusting the diameter of the nozzle, vibration frequency, polymer solution concentration, and flow rate. Ultrasonic atomisation and supercritical fluid methods are characterised by simple operation, aseptic processing, and continuous production. Furthermore, linear drug release can be achieved by optimising the experimental conditions. Among them, the ultrasonic atomisation process is suitable for the construction of microspheres loaded with biological macromolecular drugs such as polypeptides due to their relative mildness.

Additionally, inkjet printing technology and microfluidic technology have become popular in recent years. The microsphere construction of inkjet printing technology is similar to how inkjet printers work. The polymer solution was initially extruded drop by drop using a drop-on-demand piezoelectric inkjet printer, and finally, microspheres were formed at the tip of the nozzle. The microspheres prepared by the method have uniformity and large yields and are easily industrialised. However, its scope of application is limited by the difficulty in dispersing higher-viscosity fluids, which requires high equipment. Micro-fluidic technology is based on a microfluidic chip, which uses a specific channel structure inside the chip to promote the formation of a droplet or laminar flow of the solution. The preparation principle of this method is similar to the emulsification cross-linking method. However, the difference is that the two processes of emulsification and cross-linking are independent.

Therefore, through the different ways of material selection and preparation of load can be achieved microspheres drug targeting delivery, also has a controlled release rate and improves the bioavailability of characteristics. In addition, the surface of the microspheres can also be modified to confer targeting. How to improve the performance of graft, and promote the early bone union, reduce the area surrounding the incidence of infection, is to grow to repair (jaw, femur, bone and cartilage) in the field of new research hotspots. For example, the use of PTMC or poly(L-lactic acid) (PLLA) to prepare biodegradable microspheres avoids the use of stainless steel and subsequent osteoporosis caused by secondary surgery.[126] To prevent implant infection and promote healing, antibiotics or growth factors are often loaded into microspheres during treatment.

Bone Regeneration Microsphere Scaffolds

Due to the tiny and rigid shape, microspheres can be easily assembled to form microsphere scaffolds imitating the porous structure of bone tissue. Currently, microsphere scaffolds are mainly divided into microsphere-based and incorporated microsphere scaffolds. Microsphere-based scaffolds are mainly constructed by microspheres, which can be categorised into sintered scaffolds and microsphere-injected scaffolds. For the incorporated microsphere scaffolds, its main preparation is mainly divided into microsphere synergistic scaffolds and microsphere co-mingled printing ink preparation scaffolds, the combination of scaffolds is mainly through the microsphere loading in the existing scaffold system to solve the shortcomings of the incompatibility of some of the microsphere-loaded drug properties and mechanical strength.[10, 11] The scaffolds prepared by co-mixing microspheres into the printing ink enable the microspheres to be immobilised inside the scaffolds to form a multistage pore network structure. In this section, the performance requirements, preparation methods and applications of microsphere scaffolds are described.

Performance requirements for bone regeneration microsphere scaffolds

Bone repair scaffolds are inspired by the structure and physiology of natural bone,[127] and synthetic polymer microsphere scaffolds, as a kind of tissue engineering scaffolds with adjustable physicochemical properties, also need to be adapted to the ideal bone scaffold performance (**Figure 5**). It is well known that the appropriate pore size can effectively promote cell proliferation and nutrient transport and metabolism. Nanoscale micropores tend to be conducive to the binding and adsorption of bioactive factors, 10–100 μm pores are suitable for capillary growth, 40–100 μm pores are beneficial to the growth of non-mineralised tissues, and 150–800 μm micropores are the main channels for the metabolism of nutrients in bone tissues.[127-131] Therefore, utilising the interactions between microspheres can effectively improve the microporous channels within the scaffold as well as the overall porosity size. The unique surface structure of the microspheres makes it possible to effectively improve the mechanical properties of the scaffolds under the interaction between the microspheres and the microsphere scaffolds, especially for the sintered microsphere scaffolds, where the porosity and mechanical strength of the scaffolds are controlled by the degree of bonding between the sintered microspheres. For mechanical properties, meeting a

compressive strength of 2–12 MPa and Young's modulus of 7–30 GPa can ensure that the scaffolds can be filled in cancellous bone defects.[132, 133] In addition, disease and ageing are the main causes of bone tissue loss and individualised differences in load-bearing in bone tissue defects require pre-simulation of the required stress demands at design time.[134] The shape of artificial bone often depends on the anatomical structure of the bone defect site, along with the complexity of the disease and individual patients with different defect sites, the need for bone repair scaffolds to adapt to different defect sizes and shapes, through the injection of hydrogel and scaffolds with shape memory can be well integrated into the interface of the bone defect to meet the anisotropy of the filling. In addition, good biocompatibility, degradable behaviour and different functionalisation requirements are expected from all types of microsphere scaffolds.[10, 11]

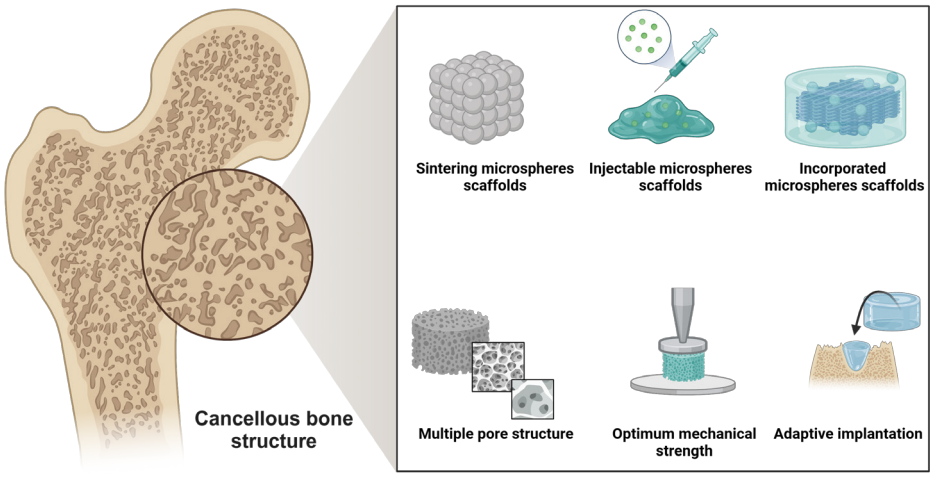

Figure 5. Construction of microsphere scaffolds based on cancellous bone structure and function. Based on the structure and properties of cancellous bone, sintered microspheres, injectable microspheres, and incorporated microspheres can be prepared. The three kinds of scaffolds can be adjusted according to the performance requirements of multiple pore structures, mechanical strength, and adaptive behaviour. Created with BioRender.com.

Therefore, different assembly methods of microsphere scaffolds can enable the mechanical properties, degradation behaviour and physicochemical properties such as drug encapsulation of microsphere scaffolds to meet different clinical needs. In addition, the microsphere monomer itself as an emerging micron-sized material will also have different performance advantages through different preparation methods, which will also greatly increase its application scope (**Table 2**).[135-154]

Sintered microsphere scaffolds

Preparation of sintered microsphere scaffolds

To significantly improve the mechanical properties of microsphere scaffolds and effectively improve the pore size of small and large micropores, two studies developed microsphere scaffolds molded by sintering.[11, 155] This sintered scaffold preparation technique fuses single microspheres to form a cancellous-like bone scaffold structure with a through-pore structure. The current sintering techniques for microsphere scaffolds include thermal sintering, carbon dioxide sintering, solvent sintering, and laser-assisted sintering.[11, 12] Among them, the basic principle of thermal sintering and laser sintering is to increase the glass transition temperature of polymer microspheres to realise the sintering, while solvent sintering and carbon dioxide sintering are two sintering methods using the addition of dichloromethane-type organic solvents to promote the fusion of microspheres and change the pressure of subcritical carbon dioxide (CO_2) for the fusion, respectively.[156, 157] In addition, the performance of sintered microsphere scaffolds is mostly affected by some common factors during the preparation process, such as transition temperature, viscosity, molecular weight, crystallinity and surface tension.[12] In this section, we will introduce different types of microsphere scaffold sintering methods and elaborate on the performance changes brought about by their sintering influencing factors (**Figure 6A**).

Research progress on sintered microsphere scaffolds

Thermal sintering: Thermal sintering involves loading fabricated microspheres into a specific mold and heating them to a specific temperature above the glass transition temperature (transition temperature) of the microsphere polymer material for a few hours, which melts the surface layer of the microspheres to allow them to bind to their neighbouring monomers, thus forming a 3D porous scaffold.[158] The sintered scaffolds are usually rated on their structural and structural properties in terms of the bone tissue and the temperature and time of sintering are often important factors. The sintering temperature and sintering time during the sintering process are often important factors, and different sintering times and temperatures can alter the fusion process between individual microspheres, leading to changes in pore size, compression modulus, and other properties of the microspheres.

Table 2. Application of polymer microsphere scaffolds in bone repair

Type of scaffold	Material	Loaded component	Performance advantage	Reference
Sintered microsphere scaffold	PLA-TMC, chitosan	/	PLA-TMC/chitosan microsphere scaffolds exhibited excellent biocompatibility as they not only managed to improve adhesion and proliferation of MC3T3-E1 cells but fulfilled enhancement of ALP activity as well	135
	PTMC, β-TCP	Dexamethasone	The water absorption of the scaffolds can enhance the penetration of nutrients and the excretion of waste, which are beneficial to support the growth of the tissue. Scaffolds delivered dexamethasone in a controlled release manner for sustained release to promote tissue growth	136
	PLGA, nano-hydroxyapatite	DNA	They are highly cytocompatible and can serve as bioactive scaffolds for the release of DNA-loaded calcium phosphate nanoparticles for local gene transfection	137
	PCL	/	Integrated osteochondral scaffolds made of sintered PCL microspheres can provide effective mechanical support and similar compressive strength with native osteochondral tissue. Promotes vascular regeneration and cartilage reconstruction	138
	PLGA, calcium carbonate, hexagonal mesoporous silica	/	Compared with HMS/PLGA scaffolds, the proliferation of MSCs cultured on CC/HMS/PLGA scaffolds was enhanced. When cultured on the CC/HMS/PLGA scaffolds, MSCs also showed significantly enhanced ALP activity and higher calcium secretion compared with HMS/PLGA scaffolds	139
	Biphasic calcium phosphate	/	Biphasic calcium phosphate scaffolds fabricated by indirect SLS printing maintain the physicochemical properties of biphasic calcium phosphate and possess the capacity to recruit host precursor cells to the defect site and promote endogenous bone regeneration possibly via the activation of ERK1/2 signalling.	140
Injectable microsphere scaffold	GelMA	BMSCs, HUVECs	Development of vascularised bone-like tissue with high levels of OCN and CD31	141
	GelMA, bisphosphonate	Mg^{2+}	Both *in vivo* and *in vitro* experimental results revealed that the magnet-inspired Mg^{2+}-capturing composite microspheres are beneficial to osteogenesis and angiogenesis by stimulating osteoblasts and endothelial cells while restraining osteoclasts, and ultimately effectively promoting cancellous bone regeneration	142
	GelMA	BMSCs	The freeze-dried microspheres of particle size 300 μm and pore size 50 μm rapidly adsorbed murine BMSCs and maintained their viability and osteogenic potential *in vitro*. In addition, the cell-loaded porous microspheres promoted tissue regeneration when injected locally into a murine bone defect model	143
		MSCs, PDGF-BB	*In vitro* and *in vivo* experiments validated that the living GMs exhibit superior secretion properties and anti-inflammatory efficacy and can attenuate osteoarthritis progression by favouring the adherent microenvironment and utilising the synergistic effect of exogenous and endogenous MSCs	144
	PLGA, chitosan	Kartogenin, MSCs	*In vivo* and *in vitro* experiments show that PLGA-chitosan microspheres have a high cell-carrying capacity up to 1×10^4 mm^{-3} and provide effective protection of MSCs to promote their controlled release in the osteoarthritis microenvironment. Simultaneously, kartogenin loaded inside the microspheres effectively cooperated with PLGA-chitosan to induce MSCs to differentiate into chondrocytes	145
	HAMA, PEG	Kartogenin, hydrogenated soya phosphatidylcholine	MHS@PPKHF forms a buffer lubricant layer in the joint space to reduce friction between articular cartilages while releasing encapsulated positively charged PPKHF to the deep cartilage through electromagnetic force, facilitating visualisation of the location of the drug via fluorescence. Moreover, PPKHF facilitates the differentiation of BMSCs into chondrocytes, which are located in the subchondral bone. In animal experiments, the material accelerates cartilage regeneration while allowing monitoring of cartilage layer repair progression via fluorescence signals	146
	HAMA-SA, ChSMA	Chemokines, macrophage antibodies, and engineered cell membrane vesicles	*In vitro* experiments demonstrated that immune cell-mobilised hydrogel microspheres had excellent macrophage recruitment, capture, and reprogramming abilities. Pro-inflammatory macrophages can be transformed into anti-inflammatory macrophages with an efficiency of 88.5%. Animal experiments also revealed a significant reduction in synovial inflammation and cartilage matrix degradation of osteoarthritis	147
	Thiolated hyaluronic acid	UCMSC-derived exosomes	Extend the retention of exosomes *in vivo*. The higher enrichment of exosomes on the cartilage surface achieved by chondrocyte-specific targeting peptide significantly improved the therapeutic effects on ageing chondrocytes and promoted the repair of articular cartilage due to their higher efficiency	148
Incorporated microsphere scaffold	GelMA, nano-hydroxyapatite, chondroitin sulfate A	/	Cell adhesion, proliferation and all-round migration on the scaffold reflected the favourable biocompatibility, as well as proved that the embedded microspheres acted as bridges to facilitate the communication of cells and active factors. The expression of biological factors *in vitro* and the recovery of animal skull defects *in vivo* demonstrated that G10-F@Mc scaffolds could induce osteoblastic differentiation of BMSCs and accelerate bone repair	149
	GelMA	MSCs	The osteo-callus organoids acting as microniches led to efficient ectopic bone formation and contributed to rapid in situ bone regeneration within 4 weeks in large bone defects. New bone formation under the implantation of osteo-callus organoids exhibited a temporal-forward healing phase which stepped over the chondrogenesis	150
	PLGA, nano-hydroxyapatite	Icariin	The PCL/nano-hydroxyapatite scaffold showed sustained release of icariin as the PCL degraded. The released icariin promoted the osteogenic differentiation of MC3T3-E1 cells. Consistently, *in vivo* studies showed that the icariin-releasing composite scaffolds promoted calvaria bone healing	151
	Collagen, chitosan, hyaluronic acid, PLGA	Kartogenin	Compared with the surface layer and transitional layer scaffolds group, the results of the dual-layer biomimetic cartilage scaffold group showed that the defects had been filled, the boundary between new cartilage and surrounding tissue was difficult to identify, and the morphology of cells in repair tissue was almost by the normal cartilage after 16 weeks	152
	PLGA, SF, HAP	BMSCs, naringin	The SF/HAP scaffold with naringin microspheres could positively regulate the osteogenic differentiation of BMSCs and promote the differentiation of BMSCs into osteoblasts. Naringin promotes fracture healing through the PI3K/Akt signalling pathway	153
	α-TCP, gelatin, zinc-doped bioglass	/	The long-term release of Zn^{2+} from zinc-doped bioglass can effectively upregulate the expression of Runx-2, and OCN for promoting osteogenic differentiation of BMSCs. Mg-GMS can regulate the release time and speed of Mg^{2+}, and effectively activate the expression of VEGF, and NGF to promote the reconstruction of the neurovascularisation network. The 3D-printed scaffolds provided mechanical support and interconnecting pore structures	154

Note: 3D: three-dimensional; Akt: protein kinase B; ALP: alkaline phosphatase; BMSC: bone marrow-derived mesenchymal stem cell; CC: cellular component; ChSMA: chondroitin sulfate methacrylate; ERK1/2: extracellular signal-regulated kinase 1/2; G10-F@Mc: a scaffold external frameprepared by using G10 bio-ink (GelMA: nanohydroxyapatite: lithium phenyl (2,4,6-trimethylbenzoyl) phosphinate = 10: 21: 0.2) with microspheres; GelMA: methacrylate gelatin; GM: GelMA hydrogel microsphere; GMS: gelatin microsphere; HAMA: hyaluronic acid methacrylate; HAMA-SA: streptavidin grafted hyaluronic acid methacrylate; HAP: hydroxyapatite; HMS: HAMA-SA and ChSMA microsphere; HUVEC: human umbilical vein endothelial cell; MHS: microfluidic hyaluronic acid methacrylate sphere; MSC: mesenchymal stem cell; NGF: nerve growth factor; OCN: osteocalcin; PCL: polycaprolactone; PDGF-BB: platelet-derived growth factor BB; PEG: poly(ethylene glycol); PI3K: phosphoinositide 3-kinase; PLA: poly(lactic acid); PLGA: poly(lactic-co-glycolic acid); PPKHF: polyhedral oligomeric silsesquioxane linked with PEG, kartogenin, hydrogenated soya phosphatidylcholine, and fluorescein; PTMC: poly(trimethylene carbonate); SF: silk fibroin; SLS: selective laser sintering; TMC: trimethylene carbonate; UCMSC: umbilical cord-derived mesenchymal stem cell; VEGF: vascular endothelial growth factor; α-TCP: α-tricalcium phosphate; β-TCP: β-tricalcium phosphate.

Thermal sintering is usually used to improve the mechanical properties of the scaffolds. Shahin-Shamsabadi et al.[159] first prepared PCL/bio-glass microspheres by single-emulsion solvent evapouration, and then sintered the sintered microsphere scaffolds by laying them flat in specific molds and sintering them for 100 minutes at 64.5°C. Bio-glass, which has a similar composition to animal bone, significantly improved the mechanical properties of the microsphere scaffolds, reaching a maximum compression modulus of 47.05 MPa, and also showed viscoelastic properties closer to the nature of bone tissue. It is worth noting that thermally sintered microspheres are usually compounded using synthetic polymers such as PLGA, PCL and inorganic salts, the main reason being that the inorganic salts are usually stable at high temperatures and can be used to stabilise the microsphere scaffolds. The main reason is that inorganic salts are usually stabilised at high temperatures and provide good mechanical properties for the scaffolds, and the choice of bioglass for the composite can also alleviate the acidic degradation of PCL to a certain extent and neutralise the acidic ions generated by PCL. Jose et al.[160] customised pinned and threaded macroporous bone scaffolds with high mechanical strength by doping hydroxyapatite (HAP) into PLGA microspheres, followed by thermal sintering, which interconnected the microspheres as observed by scanning electron microscopy (**Figure 6B**). In addition, immunofluorescence assays revealed significant upregulation of type I collagen and osteocalcin protein expression by co-culturing PLGA/nanohydroxyapatite (nHAP) microsphere scaffolds with BMSCs for 14 and 28 days, indicating excellent bone-enabling behaviour of the scaffolds (**Figure 6B**).

Solvent sintering: Solvent sintering is a type of sintering that can often be divided into the following categories: solvent vapour sintering, weak solvent sintering, and solvent/non-solvent sintering.[161] The most common solvent vapour sintering method is to allow the solvent to vapourise and diffuse into the microspheres so that the transition temperature of the microspheres can be further reduced to facilitate the fusion of the microspheres. The material and size of the microspheres affect the structure of the scaffolds, and in practice, the sintering rate is often limited to when the microspheres are saturated. After saturation occurs, the sintering rate is dramatically accelerated.[162] It is important to note that it is possible to mount pharmaceuticals or other bioactive molecules inside the sintered scaffolds for tissue engineering repair, and this is something we need to work on. Another solvent sintering method is weak solvent sintering, which involves first stacking microspheres inside the device mold and then reducing the transition temperature if the weak solvent is used.[163] It is important to pay attention to the duration of solvent immersion on the software, which affects the degree of adhesion of the microspheres and thus the degree of sintering. In general, the compression modulus of the scaffold increases as the degree of sintering increases.[164] The final method is solvent/non-solvent sintering, also known as dynamic sintering, in which the solvent surrounds the exterior of the microspheres and subsequently causes a loosening and swelling of the polymer, which then binds to neighbouring microspheres.[165] The advantages are that sintering can be performed from a wide range of polymeric materials and the polymer requirements are low, and different structural properties of the scaffolds can be achieved by varying the solvent/non-solvent concentration. He et al.[103] prepared chitosan-coated PTMC/PLLA/oleic acid (OA)-modified scaffolds to improve the biological and drug delivery capabilities of the microsphere scaffolds. HAP/vancomycin hydrochloride microsphere scaffolds.[103] The incorporation of PLLA and OA-HA significantly improved the mechanical and surface properties of PTMC microspheres, and the mechanical properties of PTMC/PLLA/OA-HA/vancomycin hydrochloride scaffolds analysed by finite-element simulation were significantly superior to those of PTMC/PLLA and PTMC/OA-HA stents (**Figure 6C**). In addition, attaching the chitosan coating in OA-HA indicated that the proliferation of osteoblasts on the surface was stimulated by adhesion.

Carbon dioxide sintering: The traditional gas foaming method is not suitable for the preparation of microsphere scaffolds due to the confined surface of the scaffolds and the discontinuity between the pores, which can be improved by using the subcritical CO_2 sintering method. Through subcritical sintering, the CO_2 gas can reach equilibrium at a lower pressure, so that the overall swelling level and plasticising ability of the material can be maintained at a lower level, which allows the microspheres to maintain their original morphology and adhere to neighbouring microspheres, and the post-processing treatment without washing and freeze-drying can remove the residual polymers efficiently, which improves the scaffold's biocompatibility.[166] Bhamidipati et al.[167] prepared PCL and PLGA microspheres by emulsion solvent evapouration. PLGA microspheres were exposed to 364 psi (~25 bar = 2500 kPa) CO_2 absolute pressure for 1 hour at room temperature PCL scaffolds were obtained by exposing them to 690 psi (47.6 bar = 4760 kPa) absolute pressure for 4 hours at 45°C. The scaffolds obtained had similar porosity and morphology, however, in the osteogenic differentiation assay, the alkaline phosphatase activity of PLGA microsphere scaffolds was significantly higher than PCL microsphere scaffolds, suggesting that the scaffolds are more biocompatible than PCL microsphere scaffolds. higher than that of PCL microsphere scaffolds, indicating that PLGA microsphere scaffolds at lower CO_2 pressures have better osteogenic differentiation properties also suggesting that PLGA may be the material of choice for the CO_2 sintering method.[168] Additionally, subcritical CO_2 sintering with its mild molding method can add more bioactive factors, Singh et al.[169] sintered poly(propyleneglycolide-co-glycolide) microsphere scaffolds containing cells from MSCs under 15 bar (1500 kPa) CO_2 pressure at 25°C, allowing the scaffolds to have customisable shapes by sintering in different molds, and the average pore size of the scaffolds was measured to be around 40–70 μm, and the elastic modulus of the scaffolds ranged from 71 to 196 kPa. In addition, the production of GAG signs in Safranin-O staining and signs of cartilage-like matrix formation in the biochemical analyses give these CO_2 sintered microspheres an advantage in cartilage and skin repair.

Laser sintering: Laser sintering is the layer-by-layer generation of 3D scaffolds through computer-aided design data-slicing preprocessing. The formation of laser-sintered microsphere scaffolds is due to the high-temperature laser beam raising the surface of the microspheres to transition temperature temperatures at which they can be fused. In addition to the sintering temperature, the performance of the sintered

microspheres is largely influenced by predefined parameters, such as the laser power, the scanning speed of the laser, and the layer height of the computer-aided design-designed scaffold.[170, 171] However, the patient-specific customisation and reproducible and rapid preparation of the scaffolds, the applicability of a wide range of biofunctional materials and the applicability of non-toxic solvents are potential advantages of selective laser sintering scaffolds. Lin et al.[172] prepared HAP/PDLLA composite microspheres by emulsification solvent evapouration method, and then prepared HAP/PDLLA composite microsphere scaffolds by optimising the selective laser sintering sample parameters, the experiments investigated the effects of preheating sintering temperature, laser power and scanning speed on the molding effect of microsphere scaffolds, respectively. The experimental results show that the preheating temperatures of 40, 50, and 60°C, and 50°C in the preheating temperature settings make the sintered scaffolds have the ideal layer thickness and do not cause excessive melting between the microspheres and lead to scaffold collapse, and through the tensile test and the morphology of the microsphere scaffolds and other studies. Similarly, at this time with stable size and intact structure, and their microspheres remained spherical without being destroyed, among the three influencing factors laser power > scanning speed > layer thickness. Wang et al.[138] In response to osteochondral defect repair, by constructing small PCL microspheres and large PCL microspheres at the microscopic level to mimic the chondral region and subchondral bone region, respectively. At the macroscopic level, integrated scaffolds (**Figure 6D**) with three different channel modes, namely, non-channel, continuous channel, and discontinuous channel, were prepared by selective laser sintering technology, and the results showed that the special hierarchical structure of the discontinuous channel scaffolds is a feasible scaffold construction method to ensure that the scaffolds have self-adaptive compressive strength and graded connecting porosity while realising the regeneration of the cartilage and reconstruction of the subchondral bone. It is a feasible way to construct scaffolds.

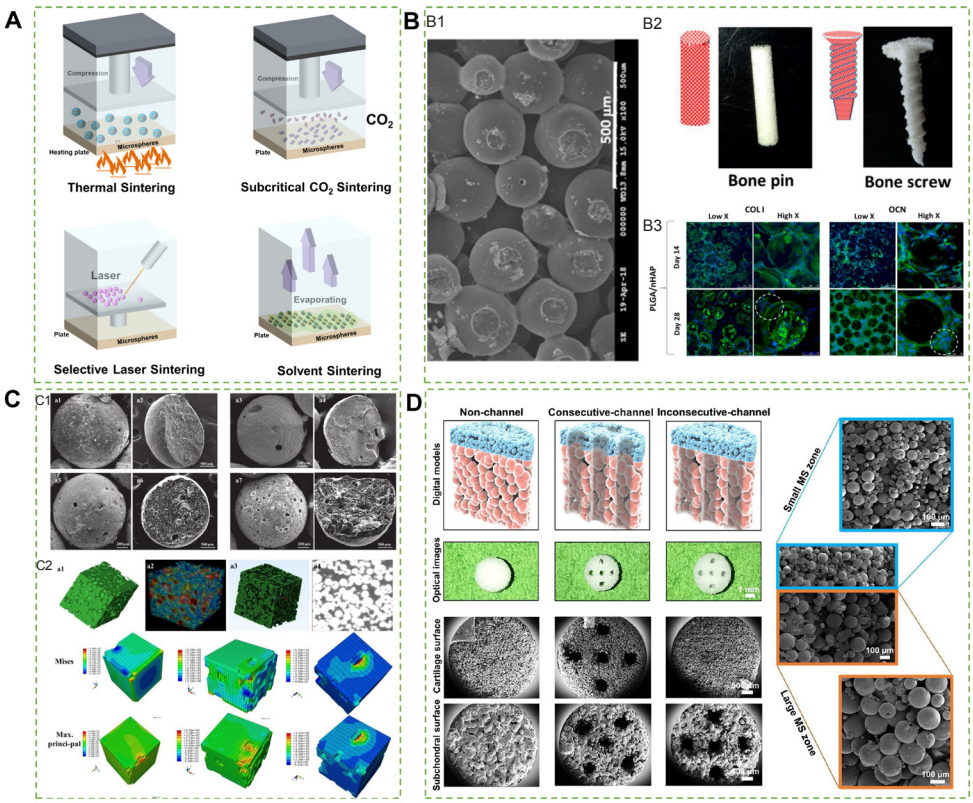

Figure 6. Preparation and application of sintered microspheres (A) Different ways of preparing sintered microsphere scaffolds: thermal sintering, solvent sintering, carbon dioxide sintering and laser sintering. Created with Microsoft PowerPoint 2016. (B) Sintered scaffolds prepared from PLGA porous microspheres. Reprinted from Jose et al.[160] (B1) Scanning electron microscopy of PLGA sintered microsphere scaffolds. (B2) Schematic and photographs of sintered PLGA/nHAP pinned and threaded bone grafts. (B3) Staining showing co-culture of PLGA/nHAP scaffolds with BMSCs for Col I and OCN protein expression. (C) CS-PTMC/PLLA/OA-HA/VH sintered microsphere scaffolds. Reprinted from He et al.[103] Copyright 2020, Elsevier Inc. (C1) Microsphere SEM. (C2) Finite element analysis of microsphere scaffolds. (D) Characterisation of three integrated scaffolds: non-channel, continuous channel, and discontinuous channel. Reprinted from Gu et al.[138] Copyright 2022, Acta Materialia Inc. BMSCs: bone marrow-derived mesenchymal stem cells; Col I: type I collagen; CS-PTMC: chitosan-coated polytrimethylene carbonate; HA/VH: hydroxyapatite/vancomycin hydrochloride; nHAP: nanosized hydroxyapatite; OCN: osteocalcin; PLGA: poly(lactic-co-glycolic acid); SEM: scanning electron microscopy.

Injectable microsphere scaffolds

Preparation methods

Tissue engineering scaffolds based on injectable microspheres have been used for cartilage, arthritis, and nerve repair, and their simple maneuverability and excellent drug loading properties enable in situ regeneration of bone defects (**Figure 7A**). This section focuses on advances in the application of polymer and hydrogel injectable microsphere scaffolds. Hydrogel microspheres are micro- or submicron-sized gel dispersion systems with the advantages of injectability, small particle size, large specific surface area, and strong host integration. Based on the high environmental conditions required for the curing of hydrogel microspheres, electrospray and microfluidic technologies are the mainstream technologies for their preparation.[143] Microfluidics is one of the most effective fluid control techniques, which allows precise control of multiphase fluids. This method allows the preparation of microspheres with highly uniform particle size and the precise adjustment of the geometrical characteristics and composition of the microspheres. As a result, monodisperse, size-controlled, function-specific, and form-specific engineered microspheres can be constructed.[114] Electrostatic spraying technology can utilise high molecular weight polymers to prepare microspheres with unique morphology and high encapsulation rate. In addition, this method requires less solvent and avoids the use of surfactants.[173] Conventional emulsification or phase separation methods for the preparation of hydrogel microspheres may require freeze-drying. In contrast, the emulsification-solvent evapouration method is the most commonly used technique for the preparation of polymer microspheres. The microsphere preparation method is simple and easy to perform, and most polymers are easily cured into spheres. In addition, the morphology and structure of the microspheres can be altered by controlling the preparation parameters, such as polymer molecular weight, solvent evapouration rate and drug type.[174]

Research progress on injectable microsphere scaffolds

Injectable polymer microspheres: PCL is a semi-crystalline polymer with a low melting temperature and is the most widely used and studied aliphatic polyester material. Microspheres prepared using PCL can be used as immobilisation matrices for bioactive molecule delivery. Jang et al.[175] constructed PCL hybrid microspheres with hyaluronic acid (HA) added by spray precipitation technology. Using the biological activity of HA significantly enhanced the regeneration of rat calvarial bone defects. However, one issue in designing injectable scaffolds based on PCL microspheres is how to promote the synthesis of elastic fibers and collagen. Bahadoran et al.[176] constructed PCL slow-release microspheres loaded with basic fibroblast growth factor. They embedded PCL microspheres in a hydrogel (polyvinyl alcohol/sodium alginate) system as a skin scaffold, which effectively promoted subcutaneous tissue regeneration and burn healing in rats.

PLGA has a controllable degradation rate compared to PCL and has been widely used in drug release. Yuan et al.[177] developed PLGA-based biodegradable microspheres that can control the release of Mg^{2+}. Such PLGA/magnesium oxide/magnesium carbonate microspheres provide feasibility for modulating biomineralisation, cell migration, osteogenic differentiation, and antibacterial activity. The microspheres act as injection scaffolds to enhance cell proliferation and differentiation by promoting cell migration of BMSCs. Moreover, PLGA microspheres can be used to transport living cells. To improve cartilage regeneration, Qu et al.[178] prepared BMSC-loaded open-pore PLGA microspheres.

Although polymer microspheres can be used as injectable scaffolds, they are still insufficient in terms of their plasticity at the defect site. To overcome this problem, more research has focused on composite injectable scaffolds composed of hydrogels and polymer microspheres. This approach can enhance the mechanical properties of pure hydrogels and improve the shorter residence time after hydrogel implantation. Two different states of PLGA-alginate scaffolds were prepared by García-Garcíaa et al.[179] to promote bone regeneration in osteoporosis. The two scaffolds are liquid hydrogels and solid sponges, respectively, which can be used for sustained delivery of β-estradiol and BMP-2. Among them, the solid sponge group showed a significantly high rate of bone formation. Similarly, the controlled release of active substances can also be achieved by encapsulating polymer microspheres in hydrogels. Lin et al.[180] encapsulated PLGA microspheres in methoxy PEG-poly(alanine) hydrogels for sustained release of growth factor TGF-β3, thereby enhancing chondrogenic effects.

Furthermore, polymer microspheres can be encapsulated in an injectable calcium phosphate cement to enhance bone regeneration. Liao et al.[181] compared the degradation performance of calcium phosphate cement encapsulating three different types of polymer microspheres. The results showed that the calcium phosphate cement group encapsulated with PLGA microspheres degraded faster and had a less inflammatory reaction, effectively promoting bone defect regeneration.

Compared with PLGA, PLLA has more robust mechanical properties. To meet the degradability and hydrophilicity required for the application, PLLA is often copolymerised with PEG to construct a triblock copolymer PLLA-PEG-PLLA. Coatings are the simplest option to improve the biocompatibility of injected microspheres with surrounding tissues after implantation. Wei et al.[182] combined PLLA-PEG-PLLA with alendronic acid to construct biomineralised microspheres and added a gelatin coating on the surface to enhance its biocompatibility. The results showed that the microspheres prepared by adding 50 μM alendronate could effectively promote osteogenic differentiation and bone defect regeneration. Due to the weak biomineralisation ability of PLLA-PEG-PLLA, an apatite layer can also be deposited on the surface. Mao et al.[183] constructed polydopamine-coated microspheres to serve as sites for surface deposition of apatite. Moreover, growth factor BMP-2 was loaded into the microspheres, so that the composite microspheres had both osteoinductive and osteoconductive properties to stimulate bone regeneration.

Currently, good biocompatibility and antibacterial ability need to be considered when designing bone defect implant materials. Loading antibiotic drugs into microspheres is

the easiest way to achieve the purpose of killing bacteria. Wei et al.[184] prepared a biodegradable PLLA-PEG-PLLA microsphere containing vancomycin and strontium-doped apatite to provide histocompatibility and antibacterial activity. Interestingly, introducing the strontium element enhanced MSC angiogenesis and osteogenic expression. Moreover, other antibacterial nanomaterials can also be loaded into the microspheres. Huang et al.[185] constructed conductive aniline tetramer-substituted polyphosphazene microspheres loaded with modified polydopamine and silver nanoparticles, which endowed the microsphere scaffolds with antibacterial and osteogenic activities. Among them, the electron-donor structure of the aniline tetramer moiety can also effectively scavenge reactive oxygen species.

Polyhydroxyalkanoate is a semi-crystalline aliphatic polyester produced by microorganisms under unbalanced growth conditions. Similar to materials such as PCL and PLLA, polyhydroxyalkanoate has good biodegradability and biocompatibility. Wei et al.[186] prepared highly open porous polyhydroxyalkanoate microspheres with diameters of 300 – 360 μm. There are channels of about 8.8 μm inside the microspheres, which have stronger osteoblast regeneration functions.

Besides the above polymer-based injectable microsphere scaffolds, there are also some multifunctional microspheres based on amino acid materials. Rudnik-Jansen et al.[187] constructed polyesteramide microspheres with the long-term release of triamcinolone acetonide. Significantly, polyesteramide microspheres effectively reduced inflammatory responses in an osteoarthritis model. Gong et al.[188] constructed porous microspheres by blending/grafting poly(c-benzyl-L-glutamic acid) with HAP. The results showed that the microspheres had higher porosity, more uniform osteogenic differentiation, and higher osteogenic gene expression performance. Fang et al.[189] exploited the electrostatic interaction between PLGA and chitosan to develop a PLGA/chitosan polyelectrolyte complex microsphere with an average pore size of 47.5 ± 5.4 μm. The microspheres produced more cartilage matrix after subcutaneous injection in animals than single chitosan microspheres. These amino acid-based injectable microspheres are less prone to rejection when implanted in animals. Bai et al.[145] prepared PLGA porous microspheres loaded with kartogenin (KGN) by a stem cell tissue engineering method (**Figure 7B1**), followed by anchoring chitosan by an amidation reaction (PLGA-chitosan@KGN), and then subsequently loaded the microspheres with stem cells by a 3D co-culture of the microspheres (**Figure 7B2**), which demonstrated good biocompatibility and promoted cartilage repair.

Injectable hydrogel microspheres: Methacrylate gelatin (GelMA) is a kind of double-bond modified gelatin, which can be cross-linked and cured into a gel by a photoinitiator under the action of ultraviolet and visible light. GelMA combines the characteristics of natural and synthetic biomaterials with a 3D structure suitable for cell growth and differentiation, excellent biocompatibility, and cellular response properties.

To treat osteoarthritis caused by reduced joint lubrication performance and continuous inflammatory response,[190] Han et al.[191] constructed cocoa-injectable hydrogel microspheres loaded with diclofenac sodium anti-inflammatory drug. Injecting the microspheres into the knee joints of osteoarthritis model rats greatly improved the lubricity and achieved sustained local drug release. Similarly, localised hyperactive inflammation in degenerative diseases may affect implant therapy. Bian et al.[192] constructed an injectable "peptide-cell-hydrogel" microsphere to regulate the ECM's metabolic balance. Meanwhile, local inflammatory cytokine storm was suppressed *in vitro* by covalently coupling APETx2 to loaded nucleus pulposus cells. Shen et al.[193] focused on the use of hydrogel microspheres to cultivate cartilage-like organs, which not only mimicked the function of natural cartilage but also facilitated the repair of host tissues. They used a microfluidic integrated system with photocrosslinking and self-assembly technologies to prepare novel Arg-Gly-Asp peptide-filament protein-DNA hydrogel microspheres, which have a uniform particle size distribution, good solubility properties, and suitable degradability (**Figure 7C1**). Arg-Gly-Asp peptide-filament protein-DNA hydrogel microspheres can up-regulate integrin-mediated cellular adhesion and local adhesion pathways, which promotes glycosaminoglycan biosynthesis, and induces BMSCs into cartilage differentiation (**Figure 7C2**). Finally, Arg-Gly-Asp peptide-filament protein-DNA hydrogel microspheres and COP were implanted into a cartilage defect model in Sprague-Dawley rats for cartilage repair function validation, which significantly accelerated cartilage regeneration and repair.

The hydrogel stability can be enhanced by cross-linking and acting as an anchoring site to incorporate more active substances. Zhao et al.[142] constructed bisphosphonate-functionalised injectable hydrogel microspheres by the coordination reaction of metal ion ligands, which can capture and release Mg^{2+} slowly. Additionally, bone targeting can also be achieved by the slow release of bisphosphonate. The microspheres can promote the remodelling of cancellous bone in osteoporotic bone defects, which is beneficial to angiogenesis and bone regeneration.

Despite the excellent properties of GelMA, hydrogel microspheres constructed using natural derivatives have also received extensive attention. Liu et al.[194] developed cell-derived ECM microspheres (bionic cartilage acellular matrix microspheres) for repairing cartilage defects. Bionic cartilage acellular matrix microspheres can be combined with microfracture surgery to treat articular cartilage lesions and direct the differentiation of bone marrow stem cells released from microfractures. Injectable hydrogel scaffolds generally have good osteoinductive properties but tend to ignore osteoconductivity. Ingavle et al.[195] embedded MSCs in composite hydrogels constructed with naturally derived polymers. The hydrogel contains two biomineralised polymer microspheres (alginate and hyaluronate). Based on the properties of loaded cells, the hydrogel can promote autologous MSC bone formation in a large animal sheep bone defect model. To address the dysregulation of oxygen homeostasis associated with large bone defects, Chen et al.[196] encapsulated oxygen-carrying nanobubbles in GelMA/heparin methacrylate microsphere macromolecular networks and constructed time-cavitated hydrogel microspheres by incorporating bone BMP-2.

The time-cavitated microspheres released high concentrations of oxygen at different temperatures and frequencies through the ultrasound effect to improve the oxygen imbalance at the defect site (**Figure 7D**), and showed good vascularisation and osteogenesis in both *in vitro* hypoxia and *in vivo* defect models. Implantation of injectable degradable microspheres can be performed directly or mixed with other plastic materials such as suspensions, colloids, and gels. The former exists as a stack of many spheres, while the latter is suspended in liquid. Both of these injectable scaffolds can be matched to the defect site after implantation. Various injectable microsphere scaffolds are made from natural or synthetic polymers and are suitable for different implantation sites. Preferred injectable scaffolds are mainly hydrogels or pastes with better controlled release capabilities and structural properties. However, all these injectable microsphere scaffolds may deflect during application. This displacement is due to the weak inter-particle interactions of the scaffolds, and thus they may migrate or even detach from the defect site under the influence of external forces after implantation. To address these problems, researchers have developed a number of glues or cross-linking agents to prevent injectable microsphere scaffolds from slipping out of the implantation site. However, these additionally used agents have specific cytotoxicity and bring more side effects. In addition, injectable microsphere scaffolds still have major drawbacks in some practical applications, such as cell infiltration and activity in the scaffold matrix, control of biomolecule delivery and release, and pretreatment for clinical use.

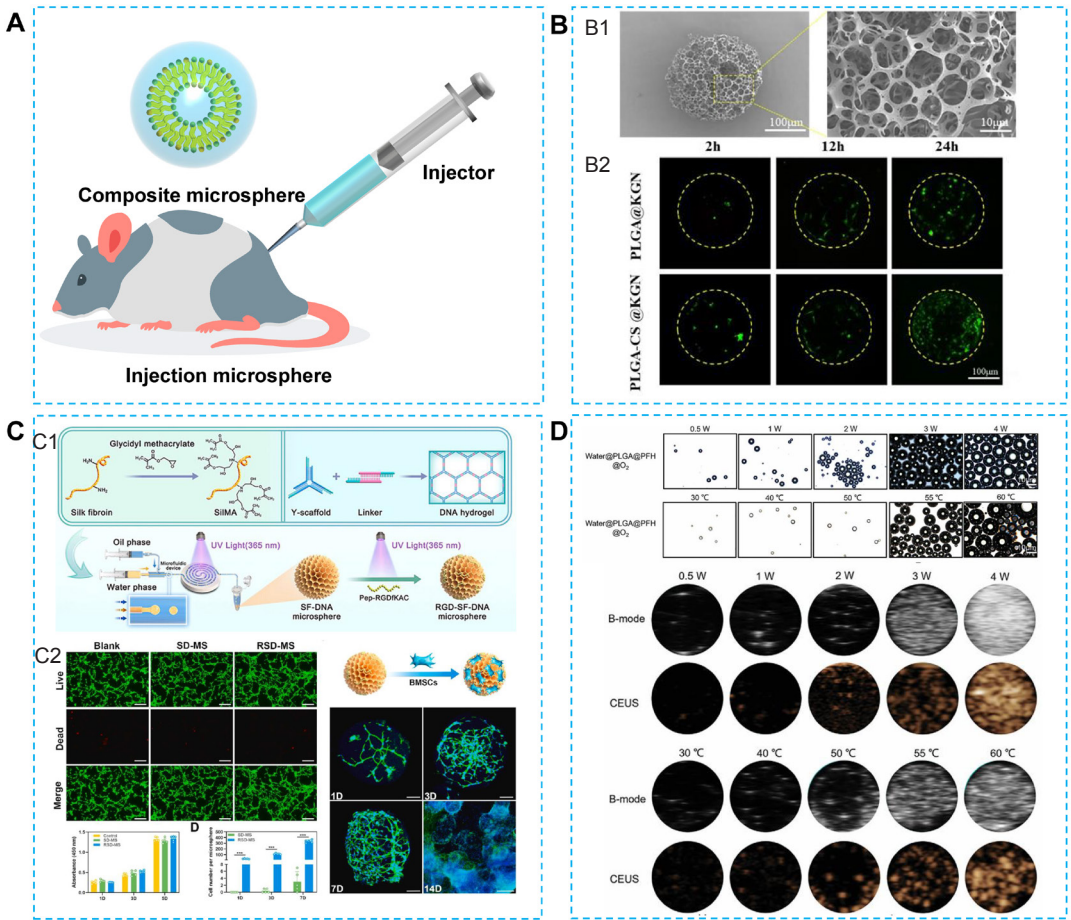

Figure 7. Preparation and application of injectable microsphere scaffolds. (A) Schematic diagram of the application of injectable microsphere scaffolds. Created using Microsoft PowerPoint 2016. (B) Promotion of articular cartilage regeneration by PLGA porous microspheres. Reprinted from Bai et al.[145] Copyright 2023, Wiley-VCH. (B1) Scanning electron microscopy of PLGA porous microspheres (B2) Stem cell amplification behaviour on the microspheres (C) Sericin-DNA hydrogel microsphere scaffolds for cartilage repair.Reprinted from Shen et al.[193] (C1) Synthesis of RGD-SF-DNA microspheres. (C2) Live/dead staining images of BMSCs and differentiation of co-cultured BMSCs into cartilage. (D) Ultrasound contrast imaging of temporalised hydrogel microspheres. Reprinted from Chen et al.[196] Copyright 2023, Wiley-VCH. BMSCs: bone marrow mesenchymal stem cell; CEUS: contrast-enhanced ultra sound mode; PFH: perfluorooctane; PLGA: poly(lactic-co-glycolic acid); PLGA@KGN: poly(lactic-co-glycolic acid)@kartogenin; PLGA-CS@KGN: poly(lactic-co-glycolic acid)-chitosan@kartogenin; RGD: Arg-Gly-Asp peptide; RSD-MS: RGD-SF-DNA hydrogel microsphere; SD-MS: SF-DNA hydrogel microsphere; SF-DNA: silk fibroin-DNA; SilMA: silk fibroin methacryloyl; UV: ultraviolet.

Incorporated microsphere scaffolds

Preparation of incorporated microsphere scaffolds

Although sintered scaffolds have good mechanical properties, their theoretical porosity is generally much lower than that of cancellous bone, and their solid structure is usually unable to rapidly release the loaded active drug molecules, thus affecting the growth of small blood vessels and granulation tissues during the growth cycle of the bone tissue and thus slowing down the bone healing.[197] Injectable microsphere scaffolds, while having good biocompatibility, can effectively carry out the loading of stem cells and active factors to realise the in situ growth of bone tissues in the defect site, but their low mechanical properties are often unable to meet the load-bearing bone defect site filling repair.[198] Therefore, incorporated microsphere scaffolds by combining microsphere preparation technology with traditional scaffold processing technology (freeze-drying, electrostatic spinning, 3D printing, gas foaming technology, etc.) is a hot issue in current research.[199-201] Freeze-drying is the use of low-temperature freezing of solvents and low-pressure sublimation to obtain scaffolds with high porosity, and its simple preparation can easily load different kinds of materials as well as control the scaffolds' microscopic morphology. Meanwhile, different types of microspheres can be encapsulated by freeze-drying and the amount of encapsulation can be regulated to realise the controlled release of drug-active molecules. In addition, the application of microsphere scaffolds in cancellous bone repair has attracted a lot of attention. 3D printing based on computational topology design can accurately control the scaffold size, shape, scaffold structure, and filling rate, which makes it possible to fabricate porous scaffolds with bionic cancellous bone structure and controllable mechanical properties.[202] Meanwhile, integrating active factors or drugs during or after 3D printing can improve biocompatibility and promote osteogenesis. The lack of drug release rate during bone repair can be compensated by the delivery of microspheres (**Figure 8A**).

Research progress on incorporated microsphere scaffolds

Freeze-dried microsphere scaffolds: Freeze-dried combined microsphere-prepared scaffolds can better optimise cell adhesion, proliferation, and differentiation, prolong the scaffolds' degradation time, reduce the drug release rate, and better promote bone repair. PLGA microspheres are often used to deliver drugs to slow down the drug release rate due to their good biocompatibility and sustained drug delivery properties. With the continuous development of related research, various freeze-dried microsphere scaffolds for tissue repair of the skull, radius, femur, etc. were introduced. Kamali et al.[203] mixed gelatin (5% w/v) and nHAP, stored at –20°C for 24 hours, and then freeze-dried. After washing and drying, the porosity was 85.3 ± 3.4% and the pore size was 345.3 ± 9.8 μm. 28-day degradation of the PLGA/nHAP scaffold was 30.7 ± 3.1%. PLGA microspheres encapsulated with cannabidiol (CBD) increased the porosity and pore size of CBD-PLGA/nHAP. CBD release experiments revealed that both PLGA microspheres and CBD-PLGA/nHAP scaffolds sustained the release of CBD. After 25 days, 71.25 ± 3.28% CBD was released from PLGA microsphere scaffolds. Morphometric measurements of new bone tissue showed that CBD-PLGA/nHAP better promoted bone regeneration by delaying the release of CBD. The highest density of bone and cartilage tissue was observed ($P < 0.05$). In addition, radius recovery in this group was best in terms of macroscopic. Alendronate is a drug for the treatment of osteoporosis. Lee et al.[204] prepared collagen and HAP scaffolds coated with alendronate under continuous freeze-drying conditions at –80°C. PLGA microspheres with an average diameter of 29.4 ± 8.64 μm contained bone BMP-2. The scaffolds and microspheres were protective against BMP-2 and alendronate. *In vitro* and *in vivo* experiments found that microsphere-loaded collagen-HAP composite scaffolds significantly enhanced cell proliferation/differentiation and repaired the cranium with the best results. Can repair be achieved when the animal model is expanded. Zhao et al.[153] utilised filipin protein (SF)-HAP scaffolds prepared by the freeze-drying method to deliver PLGA microspheres encapsulated with naringenin (MSN) to treat rabbit femoral defects. In cellular experiments, the MSN/SF/HAP scaffolds had a high osteogenic capacity and were able to accelerate the formation of new bone. In the rabbit femur model, the bone density and bone regeneration in the MSN/SF/HAP scaffold group were found to be significantly higher than those in the control and SF/HAP groups. Clinical bone treatment often progresses from cartilage to whole bone. For example, bone tuberculosis invades cartilage. To address such problems, Wang et al.[152] prepared collagen chitosan sodium hyaluronate filipin protein scaffolds loaded with KGN-encapsulated PLAG microspheres for the treatment of cartilage injuries by freeze-drying method. Freeze-dried microsphere scaffolds can better promote bone repair. It can control the drug release from bone injury, adapt to the changes in the wound microenvironment, and meet the demand for therapeutic substances such as antibacterial, anti-inflammatory, angiogenesis and promotion of bone repair at different stages of wound healing. The solvent must be completely removed during lyophilisation. By modifying the lyophilisation cycle to obtain different pore structures, scaffolds with a more comprehensive average pore size range can be obtained. The disadvantages are that they are time-consuming and the pore size usually does not support cell proliferation.[205]

3D printed microsphere scaffolds: 3D-printed microsphere scaffold preparation can be categorised into hybrid printing and post-printing delivery. Usually, the scaffolds are prepared first and then loaded with microspheres. For post-printing delivery microsphere scaffolds, Li et al.[206] prepared PCL scaffolds using 3D printing technology, delivered PLGA microspheres encapsulated with KGN, and modified them by injecting meniscus extracellular matrix. *In vitro* synovial MSC experiments, they found that PCL scaffolds in combination with meniscus ECM could help cell migration and adhesion and maintain an excellent ability to induce cell migration. *In vivo* experiments demonstrated that PCL/meniscus ECM-KGN scaffolds had better *in situ* meniscal regeneration. The PCL/ECM-KGN microsphere group prolonged the time of KGN release and improved cartilage differentiation and matrix morphology. To solve the problem of clinical anti-infective drugs that are difficult to enter the site of bone infection. Qiu et al.[207] prepared vancomycin/PLGA copolymer microspheres and electrostatically and physically crosslinked them by their loading into β-tricalcium phosphate scaffolds. The composite scaffold materials were characterised

by their mechanical properties, biocompatibility, *in vitro* release spectra and antimicrobial properties. In the rabbit tibia infection model, composite scaffold materials showed the most significant amount of new bone formation. Meanwhile, composite scaffold materials prolonged the sustained release time of vancomycin, which has a greater potential for clinical diagnosis of localised infected bone defects.

To realise the coupling of vasculogenic and osteogenic effects, Han et al.[208] were inspired by the unique biological structure of the lotus chamber, and prepared desferrioxamine liposomes into hydrogel microspheres by microfluidics, and combined them with 3D printedβ-tricalcium phosphate ceramic scaffolds, which realised the delivery of drug-carrying microspheres in 3D printed scaffolds (**Figure 8B1**). The results showed that this multi-structured bionic model was able to grow into a large number of stem cells both on the scaffold and hydrogel microspheres (**Figure 8B2**). Simultaneous loading of desferrioxamine was able to release more than 69% of desferrioxamine within 7 days, which could serve as a basis for in-scaffold vessel formation and stem cell osteogenic differentiation. *In vivo* experiments also showed that this combined mode of action promoted the expression of related proteins such as hypoxia-inducible factor 1-α, CD31, osteopontin, and osteocalcin, which significantly shortened the bone repair time. Zou et al.[151] prepared by 3D-printing technology the PCL/PLGA microspheres/nHAP composite scaffolds with icariin (ICA)/PLGA microspheres (average particle size of about 1.28 μm, PLGA microspheres), PCL and nHAP encapsulated within the raw material package. Sustained loosening of the ICA was achieved, and new cranial bone tissues were obtained. PCL/PLGA microspheres/nHAP scaffolds were comparable to PCL/nHAP scaffolds in that ICA could increase the density of microspheres, resulting in better load-bearing capacity. *In vitro* experiments found that PCL/PLGA microspheres/nHAP scaffolds showed higher cumulative release of ICA at day 20, which could better promote bone repair. In addition, experiments on *in vivo* cranial bone defects in Sprague-Dawley rats showed that PCL/PLGA microspheres/nHAP had significant regenerative function in computed tomography, histological analysis and immunohistochemical analysis. Knee cartilage is hyaline cartilage composed of a small number of chondrocytes and a large amount of cellular matrix, and it is difficult to recover on its own after damage due to its lack of blood vessels and nerves.[209] Yin et al.[210] established a multi-channel microfluidic chip to prepare well-dispersed microspheres (**Figure 8C1**) and cell-loaded them, and then, based on the mixing of cell microsphere-containing hydrogel modular bio-inks, prepared by 3D printer loaded with cellular microspheres (**Figure 8C2**). The scaffolds not only provide the cells encapsulated within the microspheres with an ECM-like 3D microenvironment to promote their growth, proliferation, and protein secretion but also promote tissue maturation and differentiation for repairing tissue damage and functional reconstruction.

Prospects and Challenges

In this article, we summarise the application of polymer microspheres in bone tissue engineering from four aspects. In the first part, we first elucidate the mechanism of bone regeneration, describing the process of bone regeneration in terms of anatomical structure, vascular regeneration, osteoblast metabolism, and biological processes of bone regeneration, as well as the signalling pathways and regulators involved, which provide a physiological basis for the subsequent design of microsphere scaffolds. In the second part, we summarise the diverse requirements of bone regeneration grafts. Ideal bone graft properties such as osteoinduction, osteoconduction, and promotion of osteogenic differentiation are included. We then summarise the description of current clinical bone regeneration grafts in this section, including their main components and application specifications. In the third part, we summarise the design perspectives, material sources, and preparation methods of bone regeneration microsphere scaffolds, and propose the corresponding performance requirements, including mechanical properties, degradation behaviour, and drug delivery properties. In the fourth part, we start with the preparation of bone regeneration microsphere scaffolds, which are categorised into sintered microsphere scaffolds, injected microsphere scaffolds, and encapsulated microsphere scaffolds. The application of each microsphere scaffold in bone repair is also discussed, including as a carrier for cells and drugs or as a tissue engineering scaffold.

Over the past decade, microsphere preparation processes and material selection have evolved rapidly. However, there are still many challenges to preparing microsphere scaffolds that are more responsive to wound needs. Firstly, we need to explore techniques for preparing microspheres with controllable structure, controllable size, and controllable surface. Secondly, it also includes the biocompatibility and biodegradability of the microspheres themselves. For example, for bone repair, different degrees and sites of bone injury require microsphere scaffolds designed with different degradation times to promote bone regeneration. The degradation time of an ideal microsphere scaffold should match the rate of new bone formation to provide continuous support. For the time being, minimising the introduction of toxic substances as well as increasing the yield of microspheres is a challenge in the preparation of microsphere scaffolds. Finally, microspheres are a powerful tool for delivering therapeutic substances to tissue injury sites for healing promotion and have shown promising results in tissue regeneration after drug encapsulation. However, the interaction between microspheres and cells has yet to be investigated. How to achieve better drug encapsulation rate and good cellular activity is also an aspect we need to pay attention to.

In the future, how to promote the development of microsphere scaffolds toward bone regeneration, we believe that we can start from the construction of tissue engineering scaffolds, i.e., how to design the scaffolds and how to choose the active ingredients. Currently, the research hotspots include performing biomimetic simulations of the ECM, targeted modulation of osteoblast behaviour, and responsive drug release systems. In recent years, through the preparation process and material selection, we can prepare personalised microsphere scaffolds for different bone tissue defects. For example, it can be involved in the removal of metabolites during the reaction process, including CO_2, reactive oxygen species, etc.

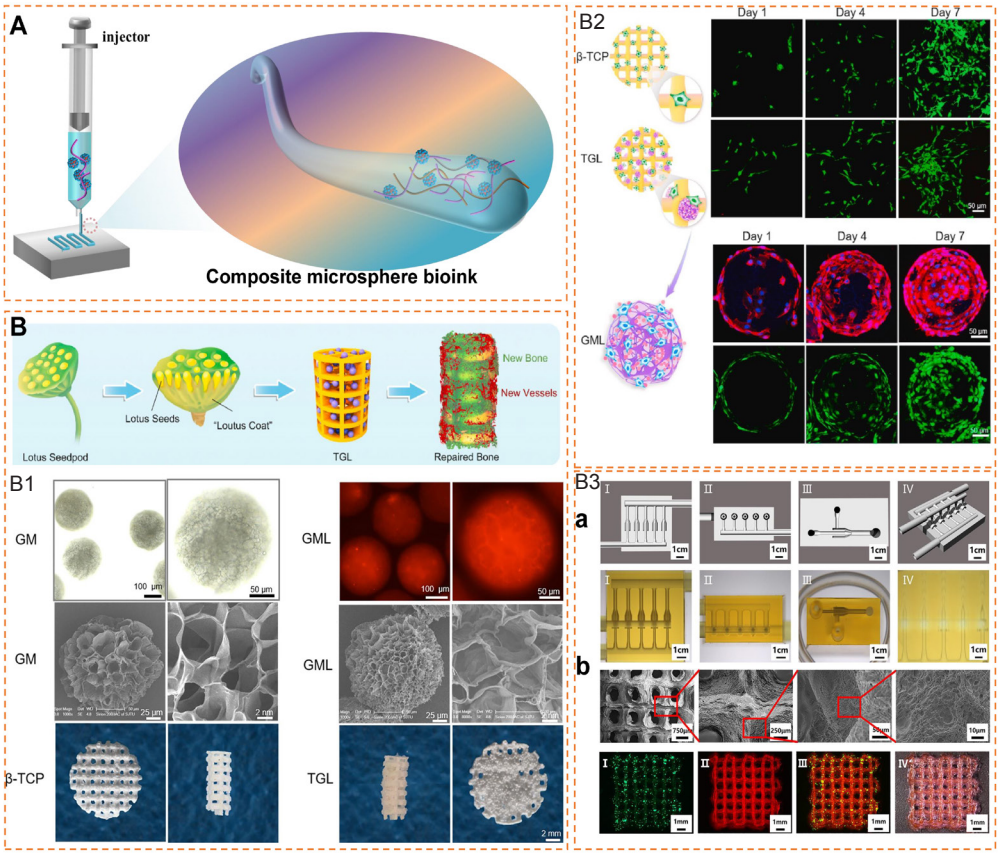

Figure 8. Preparation and application of encapsulated microsphere scaffolds (A) Preparation of bioprinting inks by blending microspheres. Created with Microsoft PowerPoint 2016. (B) Composite scaffolds imitate a "lotus" structure by incorporating microspheres with printed scaffolds. Reprinted from Han et al.[208] (B1) Bionic schematic of composite scaffolds: Liposome loaded GelMA microspheres and β-TCP scaffolds lighted images as well as electron microscopy images. (B2) β-TCP and GML microspheres Co-culture fluorescence staining images. (B3) Bioink containing microspheres synergising chondrocytes for 3D printing of multiscale composite scaffolds for cartilage repair. Reprinted from Yin et al.[210] GelMA: methacrylate gelatin; GM: GelMA group; TGL: composite scaffold incorporating GelMA microsphere@Liposome (GML) into β-TCP scaffold; β-TCP: β-tricalcium phosphate.

This review provides a comprehensive review of the research progress of microsphere scaffolds in bone repair from the perspectives of bone anatomy and bone regeneration processes. While the manuscript lacks detailed introductions for each material used in the microsphere preparation process, which may result in a certain lack of comprehensiveness, considering the main focus of the article is on producing microsphere scaffolds, it to some extent compensates for this deficiency, ensuring the overall readability of the article.

In conclusion, the development of microspheres provides a new therapeutic approach for the preparation of bone regeneration scaffolds. There are still many needs and challenges that need to be addressed in the preparation of microspheres with drug piggybacking. With the development of clinical medicine, material science and biotechnology, we believe that the above challenges can be solved through multidisciplinary cooperation to provide a good solution for bone regeneration.

Author contributions

SY led the writing of the manuscript. HW, CP and JH contributed to the writing and revisions of the manuscript. ZP and ZL contributed to the literature research and analysis, writing. JW, YH and QS contributed to the original draft and figures and tables preparation. BZ and XY contributed to the definition of intellectual content. XH, NH and HL supervised the project. XH contributed to the writing, review and editing. All authors approved the final version of the manuscript.

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Conflicts of interest statement

The authors declare no competing interests.

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