The Use of Micro- and Nanospheres as Functional Components for Bone Tissue Regeneration

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During the last decade, the use of micro- and nanospheres as functional components for bone tissue regeneration has drawn increasing interest. Scaffolds comprising micro- and nanospheres display several advantages compared with traditional monolithic scaffolds that are related to (i) an improved control over sustained delivery of therapeutic agents, signaling biomolecules and even pluripotent stem cells, (ii) the introduction of spheres as stimulus-sensitive delivery vehicles for triggered release, (iii) the use of spheres to introduce porosity and/or improve the mechanical properties of bulk scaffolds by acting as porogen or reinforcement phase, (iv) the use of spheres as compartmentalized microreactors for dedicated biochemical processes, (v) the use of spheres as cell delivery vehicle, and, finally, (vi) the possibility of preparing injectable and/or moldable formulations to be applied by using minimally invasive surgery. This article focuses on recent developments with regard to the use of micro- and nanospheres for bone regeneration by categorizing micro-/nanospheres by material class (polymers, ceramics, and composites) as well as summarizing the main strategies that employ these spheres to improve the functionality of scaffolds for bone tissue engineering.

Introduction

DESPITE EXTENSIVE EFFORTS in the field of bone tissue engineering, the use of autologous and allogeneic tissues still remains the clinical “gold standard” for bone substitution therapies. Nevertheless, the well-known drawbacks of autografts and allografts (such as short of supply, need for additional surgery corresponding to donor site morbidity etc.) are a continuous incentive to develop synthetic materials that can eventually replace auto- and allografts. Tissue engineering typically aims at reconstructing tissues by combining scaffolds composed of biodegradable biomaterials, isolated cells of the engineered tissue, or pluripotent stem cells with the addition of bioactive signals (e.g., growth factors).¹ This therapeutic strategy has shown great promise in the field of bone tissue regeneration, which focuses on restoring the functionality of diseased or damaged hard tissues caused by ageing and pathological conditions.²

In this approach, three-dimensional (3D) porous scaffolds play an essential role by providing artificial extracellular matrices that mechanically and structurally support cellular activity such as cell attachment, proliferation, and differentiation, which finally result in bone formation. To this end, these scaffolds should be biocompatible, biodegradable without producing toxic by-products, and nonimmunogenic, while exhibiting appropriate physicochemical, topographical, and mechanical properties. In addition to acting as arti-

ficial extracellular matrix (ECM), scaffolds can also serve as a reservoir of biologically active signaling molecules with a sustained presence to the physiological environment, thereby regulating cell function and triggering tissue repair.^{3–5}

However, conventional monolithic scaffolds that are typically combined with cells and growth factors are still far from leading to successful bone reconstruction in a clinical setting, mainly because of the limited control that can be exerted over biodegradation and drug delivery. For instance, direct incorporation of growth factors by adsorption onto bulk scaffolds normally leads to uncontrolled burst release on implantation and an overdose of growth factors that give rise to bone hyperplasia.⁶ A simple but effective solution for these problems has been brought forward in the 1990s by introducing microspheres as drug delivery vehicles into a continuous matrix to obtain sustained release of biomolecules without compromising the properties of the bulk scaffold.^{3–5,7}

In that way, scaffolds of higher complexity and functionality can be designed that exhibit several advantages over conventional monolithic bulk scaffolds. First of all, micro-/nanospheres have been widely accepted as a useful tool for controlled drug delivery due to their inherently small size and corresponding large specific surface area, a high drug loading efficiency, a high reactivity toward surrounding tissues *in vivo*, and a high diffusibility and mobility of drug-loaded particles.^{3–5,8,9} Second, the size and morphology of

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micro-/nanospheres allow them to quickly respond to stimuli from the surrounding environment, such as temperature,¹⁰ pH,^{8,11–13} magnetic fields,^{14,15} ultrasounds, and irradiation.¹⁰ Consequently, these spheres can serve as stimulus-sensitive delivery vehicles for biologically or chemically active agents and, subsequently, establish triggered release by responding to external stimulation³. Third, by introducing spheres as porogen, the porosity of classical scaffolding materials can be significantly improved, thereby allowing for tissue infiltration into the interior of scaffolds.¹⁶ Alternatively, mechanically weak scaffolds can be stabilized by adding micro-/nanospheres as reinforcement phase¹⁷ or crosslinking agent,¹⁸ thus providing the mechanical strength that is required for bone regeneration in load-bearing applications. Fourth, micro-/nanospheres can be used as microscopic bioreactors to induce formation of apatite crystals and subsequent mineralization of hydrogels by releasing the resultant minerals, thus forming self-hardening biomaterials for bone regeneration.¹⁹ This approach is inspired by the function of matrix vesicles in human skeletal tissues, which function as microcapsules embedded in ECM to create a compartmentalized environment for the nucleation and formation of bone minerals.²⁰ Fifth, microspheres made of cytocompatible polymers containing cell-adhesive peptide sequences can serve as a cell delivery vehicle by either cell encapsulation inside spheres or cell attachment at the exposed surface of the spheres. In that way, cell attachment sites are offered for the adhesion of anchorage-dependent mesenchymal stem cells (MSCs) such as osteoblasts.²¹ By further incorporating these cell-laden microspheres into the continuous phase of porous scaffolds or hydrogels, relatively bio-inert scaffolds can be transformed into constructs with an upgraded biological activity.²² Sixth, the spherical nature of micro- and nanospheres allows for the development of injectable and/or moldable formulations such as suspensions and (colloidal) gels to be applied by using minimally invasive surgery.^{23,24}

In view of the points just mentioned, this article addresses the most recent developments regarding the use of micro- or nanospheres for applications in bone tissue engineering. Rather than providing preparation details of micro- and nanospheres, we have categorized micro- and nanospheres for use in bone regeneration by biomaterial class (polymer, ceramic, and composites) as well as summarized the various strategies that employ these spheres as functional components for bone tissue engineering.

Material Classification of Micro- and Nanospheres

Generally, spherical biomaterials such as micro- and nanospheres should be made of biocompatible and biodegradable materials that do not release toxic degradation products. In addition, processing of these materials should be easy to enable production of micro- and nanospheres of the desired size and morphology. To meet with these requirements, polymeric, ceramic, and composite materials have been extensively investigated.

Polymeric micro-/nanospheres

Polymeric microspheres were first introduced for biomedical applications in the 1970s, initially as a drug delivery system based on polymers derived from lactic acid. Since

then, polymers have evolved as the material class that is most frequently used as a drug delivery vehicle in tissue regeneration, primarily because of their ease of processing and versatility with regard to the control over physico-chemical properties (such as their degradability).

Natural polymers are of great importance for bone tissue engineering, basically due to their intrinsic biocompatibility and biodegradability. This class of polymers is often favored more than synthetic ones due to their abundant side groups in their molecular chains that allow for further functionalization. Further, natural polymers such as collagen and gelatin contain motifs such as RGD (Arginine-Glycine-Aspartic acid) sequences, which can modulate cell adhesion, thereby improving the cellular behavior compared with polymers that lack these cell-recognition sites. Although natural polymers often possess inherent cues for directing stem cell fate, their biological activity can be lost during processing, which may induce an immune response. On the contrary, many synthetic polymers can be considered as neutral biomaterials that can be functionalized to desire to render them instructive by promoting stem cell differentiation. Typical advantages of synthetic polymers include ease of manufacturing and modification, reasonable costs, sufficient supply, no risk of disease transfer, and strong control over polymer properties such as molecular weight and corresponding degradation rates.²⁵ Additionally, the explicit definition of the polymeric composition and structure provides the resulting micro-/nanospheres with tailorable morphological and physiochemical properties that are beneficial for large-scale preparation and application.

Collagen. Collagen is the most extensively investigated natural polymer since the ECM of many mesenchymal tissues (including bone and cartilage) that are mainly composed of collagen as organic phase. Collagen is an attractive candidate material for bone regeneration due to its excellent biocompatibility, desirable biodegradability, and negligible immunogenicity.^{26,27} Collagen microspheres have been developed by using emulsification methods,^{28,29} in which homogenization of a water-in-oil emulsion is followed by gelation of collagen droplets resulting in spherical collagen. The resulting microspheres have been used as microcarriers for bioactive factors in bone regeneration,^{30–33} which showed controllable drug release kinetics by tailoring the collagen crosslinking density or by introducing bridging forces (such as electrostatic interactions) between biomolecules and the microsphere polymer network.^{30,32}

Nevertheless, only limited progress in collagen microspheres for tissue engineering has been made most likely owing to their poor mechanical stability, suboptimal processing conditions, and possible denaturation on processing.^{26,27} To address this, Chan *et al.* developed reconstituted collagen-MSC microspheres based on the self-assembly between collagen and MSCs, which exhibited desired stability as cell delivery carriers for tissue engineering.³⁴ In a similar approach toward stabilization of collagen, composite microspheres have been developed by blending collagen with other polymers (e.g., agarose³⁵ and chitosan³¹) to improve gelation and mechanical properties. Apart from physico-chemical performance, collagen extracted from animals also has the intrinsic risk of causing immune reactions and/or transmitting infectious agents.³⁶ Therefore, recent

developments in the field of bioengineered materials using recombinant collagen hold great promise for tissue engineering applications, as recombinant collagen can be a safe, predictable, and chemically defined source of collagen (by tailoring the amino acid sequence) to manufacture into materials various formulations (such as microspheres).^{37,38}

Gelatin. As a derivative from collagen, gelatin has been widely used for biomedical applications.^{21,39} Superior characteristics of gelatin include beneficial biological properties comparable to collagen, ease of processing into microspheres, gentle gelling behavior, controllable degradation characteristics by tailoring crosslinking conditions, and abundant presence of functional groups that allow for further functionalization and modification via chemical derivatization. These properties make gelatin optimal for use as a delivery vehicle for drugs or proteins. Specifically, the unique electrical nature of gelatin (commercially available as both positively or negatively charged polymers at neutral pH) enables gelatin to encapsulate bioactive molecules by forming polyion complexes.^{21,40} In addition, biomolecules can be loaded on gelatin micro-/nanospheres by diffusional loading after preparation of the microspheres, thus separating the crosslinking step from the drug loading step. As a result, controlled and sustained release kinetics can be obtained by fine-tuning the properties of the polymeric network without exposing biomolecules to harsh conditions, such as organic solvents that can deactivate highly sensitive agents such as growth factors.^{24,41–44} These growth factor-laden gelatin microspheres also function as injectable fillers for the treatment of osseous defects, which have been proved to support cell attachment, proliferation, and differentiation, and, subsequently, induce bone regeneration.^{43,45}

On the other hand, gelatin nanospheres have become increasingly attractive as particulate carrier systems owing to their high drug loading efficacy, ease of surface modification, and high uptake by cells.^{46,47} Gelatin nanospheres can be prepared by using emulsion⁴⁸ or desolvation^{49–51} methods. Desolvation is a process during which a homogeneous solution of charged macromolecules undergoes phase separation, and nanoparticles can be formed by following a nucleation-particle growth mechanism.^{49–51} Interestingly, injectable colloidal gels using gelatin nanospheres as building blocks have been recently developed in our group that are characterized by high elasticity, excellent handling properties, ease of functionalization, and cost effectiveness, which shows a great potential for tissue engineering (see Directed assembly section).⁵²

Despite the favorable properties, critical concerns of gelatin are related to its potential to induce immunogenic responses and the poor control on its physicochemical behavior due to the animal origin. To overcome these drawbacks, microspheres made of recombinant gelatin with well-defined and tunable molecular weights, amino acid sequences (such as RGD peptides), and isoelectric points have been recently developed by using emulsion methods.⁵³

Fibrin. Fibrin can be prepared by combining fibrinogen with thrombin, which are both derived from the patient's own blood, thereby fabricating an autologous scaffold that does not induce an excessive foreign body reaction.⁵⁴ Fibrin microspheres can be prepared by using simple emulsion

methods, which have been used as a platform for high-yield isolation of MSCs.^{55,56} Moreover, these microspheres can also serve as a construct to support *in vitro* MSCs expansion and osteogenic differentiation, which consequently formed a calcified matrix embedding osteoblasts,⁵⁷ and further to induce ectopic bone formation⁵⁸ as well as reconstruction of critical-size bone defects *in vivo*.⁵⁹ However, the rapid degradation rate and poor mechanical stability of fibrin have been stated as the main limitation for bone tissue engineering.⁵⁴ To overcome this problem, composite microspheres were developed to stabilize the fibrin matrix. Perka *et al.* developed alginate/fibrin composite microspheres for cell encapsulation, which solved the problems of both alginate's shortage of bioactive sequence for cell attachment and fibrin's poor capacity for cell encapsulation.⁶⁰

Chitosan. Chitosan is a frequently used hydrophilic polysaccharide derived from chitin, which exhibits favorable physicochemical and biological properties for biomedical applications including biocompatibility, intrinsic antibacterial nature, and ease of processing.⁶¹ Chitosan-based micro-/nanospheres can be prepared by using various methods,⁶² among which emulsification has been most frequently used due to the gentle processing conditions.⁶³ These micro-/nanospheres have been widely used as drug delivery systems for pharmaceutical or tissue engineering applications because of the abundant functional groups in the chitosan polymer backbone that allow for further functionalization,⁶⁴ and the capacity of chitosan to form polyion complexes with charged proteins to obtain sustained release.^{65,66} Of particular interest are that chitosan-based microspheres have been used as injectable bone fillers, which can encapsulate osteoconductive mineral (calcium phosphates [CaP]) particles in the microspheres followed by ionically crosslinking using tripolyphosphate.⁶⁷ *In vitro* studies confirmed the cytocompatibility of these chitosan-based microsphere constructs that support MSCs attachment and their subsequent proliferation and differentiation into the osteoblastic phenotype.⁶³ Chitosan has also been processed into nanosphere formulations for targeted delivery of small biomolecules.⁶² Similar to microspheres, drugs or biomolecules can be loaded in chitosan nanospheres by either incorporation during particle preparation or incubation with preformed spheres. Chitosan nanospheres are a highly promising candidate for gene delivery due to a combination of the intrinsic advantages of nanoparticles (related to their exceptionally high specific surface area), the possibility to chemically modify polymer properties, and the capacity to form polyelectrolyte complexes with negatively charged DNA due to the positive charge of chitosan.^{62,68}

Alginate. Favorable characteristics of alginate including its biocompatibility, nonimmunogenicity, hydrophilicity, and cost effectiveness, thereby making alginate highly suitable for many applications in drug delivery and tissue engineering.^{69,70} The most pronounced advantage of alginates relates to its gentle gelling behavior by which alginate microspheres can be formed by using ionic crosslinking in the presence of divalent cations such as Ca^{2+} . This unique feature of alginate has facilitated widespread usage of alginate for mild encapsulation of sensitive biomolecules and/or cells.^{71–73} It has been shown that growth factors for bone

tissue engineering loaded within alginate microspheres display controlled release kinetics,^{74,75} whereas cells immobilized within alginate microspheres showed a positive response when used as an injectable system for regeneration of skeletal tissue.^{76,77} Another attractive feature of using alginate microspheres for bone regeneration involves its intrinsic capacity to induce calcification *in vivo* without using biological or chemical additives. In a recent study by Lee *et al.*, injectable calcium-crosslinked alginate microspheres mineralized *in vivo* by forming traces of hydroxyapatite (HAP) after being subcutaneously or intramuscularly implanted.⁷⁸ The mechanism of the *in vivo* calcification was shown to relate to calcium ions from the crosslinked alginate hydrogel that interacted with surrounding phosphate ions to form precipitated CaP.⁷⁸

On the other hand, two critical drawbacks limit alginates from further applications, that is, the poorly controllable—and often slow—degradation process on implantation in bone defects, and the lack of cell attachment sites for anchorage-dependent osteoblasts. Gamma irradiation and partial oxidation of alginate have been performed to obtain microspheres with desirable biodegradability.^{72,79} On the other hand, by combining alginates with components containing cell-recognition sites (e.g., fibrin⁶⁰) or by modifying alginate with RGD sequences,⁸⁰ alginate-based microspheres revealed a significantly improved biological response in terms of the improved adhesion, proliferation, and differentiation toward the osteogenic lineage of encapsulated cells.

Poly(α -hydroxy-esters). Poly(α -hydroxy-esters), such as poly(lactic acid) (PLA), poly(glycolic acid), poly(lactic-co-glycolic acid) (PLGA), and poly(caprolactone), are the most frequently used synthetic polymers for biomedical applications owing to their biocompatibility, hydrolytic degradation process, proper mechanical properties, ease of manufacture, and so on. Micro-/nanospheres made of poly(α -hydroxy-esters) (typically prepared by emulsification) have also been widely investigated as drug delivery vehicles or tissue engineering scaffolds with regard to bone regeneration.^{81–83} By fine-tuning their physicochemical characteristics including molecular weight, particle size and morphology, and surface properties, the biological performances of these polyester spheres can be controlled to a large extent, such as loading efficiency of drug/protein encapsulation, pharmacokinetics of drug release, and cell and tissue response after implantation. For instance, Jeon *et al.* developed heparin-functionalized PLGA nanospheres with an improved capacity for controlled growth factor delivery,⁸⁴ which showed enhanced osteogenesis of stem cells *in vitro* and extensively improved bone formation *in vivo*.⁸⁵ Noteworthy is that in addition to being delivery vehicles, PLGA micro- and nanospheres have recently been extensively investigated as building blocks to establish 3D tissue-engineered scaffolds (see Micro-/nanospheres as building blocks for bottom-up fabrication of scaffolds section).^{86,87}

The disadvantages, however, of poly(α -hydroxy-esters)-based micro-/nanospheres for the widespread use in bone tissue engineering include (i) hydrophobicity resulting in poor cell adhesion and incapability of loading hydrophilic molecules or drugs, (ii) an acidic degradation product causing inflammatory tissue response and denaturation of bioactive proteins, (iii) degradation by autocatalysis leading to

unpredictable degradation behavior, and (iv) low capacity of loading therapeutic components due to the harsh preparation process and limited penetration into the polymer network.⁸⁸

Inorganic micro-/nanospheres

With regard to potential use of polymers in bone regeneration, it should be emphasized that most biodegradable polymers lack osteoconductivity, osteoinductivity, and mechanical strength. In contrast, inorganic biomaterials such as CaP exhibit excellent biological properties and mechanical strength resulting from their similarity to the inorganic phase of native bone tissue, which have been widely accepted as materials of choice for bone repair, and fabricated into bulk or micro-/nanoparticulate bone substitutes. To the best of our knowledge, however, only limited progress has been obtained on CaP micro-/nanospheres, as opposed to numerous researches focusing on nonspherical CaP particles,^{89–91} partially because of the difficulty to process CaP into spherical shape. Still, microspheres made of CaP,^{92–94} bioglass,^{95,96} or other bioactive ceramics^{97,98} have been developed that function as delivery vehicles for growth factors⁹⁵ or as particulate bone void fillers for bone regenerative medicine.⁹⁷ A versatile methodology for the preparation of bioceramic microspheres involves droplet formation of a mixture of ceramic powders and a hydrogel solution (i.e., alginate, chitosan, gelatin etc.) followed by gelation of the polymer phase that can be subsequently removed by thermal decomposition.^{98–101} CaP microspheres with monodispersity and controllable porosity and particle size have been developed as injectable scaffolds for bone regeneration by using this method, which supported *in vitro* attachment, proliferation, and osteogenic differentiation of bone marrow stromal cells that ultimately resulted in formation of microsphere-cell clusters.⁹⁹ Green *et al.* developed calcium carbonate (vaterite) microspheres containing RGD peptide sequences, which acted as a template to stimulate mineralization and MSC differentiation *in vitro*, and augment *in vivo* bone formation in impact on bone grafting.⁹⁷

Despite advantages such as ease of manufacture, low cost of production, and beneficial biological properties, inorganic micro-/nanospheres are still far from widespread use for bone tissue engineering. Plausible reasons relate to the difficulty of controlling the degradation rate, poor control over drug delivery (often related to the high affinity of bioceramics with proteins), and the propensity for growth factors to denature during adsorption to CaP.¹⁰²

Composite micro-/nanospheres

Inspired by the hierarchical composite structure and unsurpassed functionality of native bone tissue, composite scaffolds that combine the advantages but eliminate the drawbacks of each component have gained considerable interest for bone regeneration over the past decades. By mimicking the organic/inorganic composition of bone, biodegradable polymers and bioactive ceramics have been combined to fabricate composite micro-/nanospheres that improve the biological performance of polymers as well as provide bioceramics with ease of processing and controllable degradation. Thus, composite microspheres have been fabricated by incorporating CaP into biodegradable polymers such

as collagen,¹⁰³ gelatin,^{104,105} chitosan,¹⁰⁶ and PLGA^{107,108}. These composite microspheres were shown as displaying improved performance in many aspects, such as enhanced hydrophilicity (compared with pure PLGA microspheres),¹⁰⁹ higher drug/protein encapsulation efficiency,¹¹⁰ improved cytocompatibility,¹⁰⁹ reduced biodegradation and drug release rates,¹⁰⁴ and strongly upregulated *in vitro* calcifying capability.¹⁰⁴ Further, composite nanospheres where CaP nanocrystals are incorporated into polymeric nanospheres have been synthesized by employing nanosized organic templates such as liposomes¹¹¹ or polymer nanogels.¹¹² For example, gelatin/hydroxyapatite (HAP) composite nanospheres have recently developed by biomimetically inducing HAP crystallization inside gelatin spheres under physiological conditions for applications in bone tissue engineering.¹¹³

On the other hand, copolymer micro-/nanospheres have been prepared that possess advantageous properties of both polymers. Jiang *et al.* blended chitosan into PLGA microspheres to benefit from the neutralization reaction between chitosan and acidic PLGA degradation products, thus improving *in vitro* and *in vivo* biocompatibility and osteogenicity compared with pure PLGA microspheres.^{65,114,115} Copolymer nanospheres can be obtained by using the coacervation technique⁶², in which polyelectrolyte complexation can be achieved between oppositely charged polymers. This processing method enabled the synthesis of a new class of composite nanospheres typically made of polycationic chitosan and negatively charged polymers (i.e., alginate¹¹⁶, dextran sulfate¹¹⁷, etc). These hybrid copolymer nanospheres exhibited improved physical properties (e.g., *in vivo* stability), desirable surface properties (e.g., hydrophilicity/hydrophobicity and surface charge),^{118,119} and improved pharmacological performance,¹¹⁶ thus resulting in better control compared with drug delivery. For instance, chitosan-PLGA and alginate-PLGA nanospheres have been newly developed as positively and negatively charged building blocks for a colloidal gels system that self-assemble due to electrostatic interactions. These gels exhibited proper injectability and negligible cytotoxicity to MSCs.¹¹⁹

Comparison between microspheres and nanospheres

Although microspheres (defined as having diameters between 1 and 1000 μm) have been investigated for several decades in the biomedical field, the potential of nanospheres (here defined as ranging in diameter from 10 to 1000 nm) for biomedical applications has been researched over the past decade only. Both micro- and nanospheres have gained tremendous interest as reflected in Figure 1, which shows the number of publications on the use of either microspheres or nanospheres for biomedical applications over the past decade. Although the number of publications on microspheres is steadily increasing, nanospheres have been rapidly catching up with microspheres in <10 years, thus reflecting the increasing importance of nanotechnology in biomedical research.

With regard to controlled delivery and tissue engineering applications, nanospheres exhibit specific advantages and disadvantages compared with microspheres. First of all, unlike particles at a micro-scale, which would release high local drug concentrations, nanospheres can be directly en-

FIG. 1. Number of publications on the use of micro-/nanospheres for biomedical applications in the past decade by combining keywords “microspheres and biomedical” or “nanospheres and biomedical,” respectively (PubMed).

docyotosed, thereby allowing for accumulation of nanoparticle-encapsulated drugs. In that way, a desired therapeutic effect can be obtained with lower amounts of drugs while minimizing cytotoxicity or other undesired side effects.^{9,68,120} Additionally, nanospheres are capable of passing through the smallest capillary vessels (due to their inherent small size), thus avoiding rapid clearance by phagocytes so that their effective residence time in the body can be prolonged.^{121,122} Therefore, nanospheres are now increasingly accepted as suitable tools for potential applications based on cellular delivery, including cell imaging and intracellular gene delivery to control cell differentiation/survival.^{123,124} Moreover, the exceptionally high specific surface area of nanospheres broadens the range of applications related to tissue engineering. For instance, nanospheres are more competent than larger particles to serve as delivery vehicles due to their enhanced affinity to therapeutic components and quicker response to outer stimuli from the surrounding environment. Finally, nanospheres can be used as building blocks for bottom-up synthesis of colloidal systems such as injectable gels for bone regeneration. Nanosphere-based colloidal systems generally display superior properties (i.e., higher stability and better injectability/moldability) compared with microsphere-based systems owing to higher interparticle forces.^{52,87,125,126}

Still, nanospheres also display several drawbacks compared with microspheres that are related to (i) lack of cost-effective preparation techniques for nanospheres which allow for easy upscaling without using harsh processing conditions, (ii) reduced stability under *in vitro* and *in vivo* conditions due to their intrinsic high surface area that maximizes interaction with the physiological environment, (iii) the tendency of nanospheres to aggregate into a microscale that mitigates potential benefits of the use of nanoscale particles, and, finally, (iv) unsuitability for various applications in bone tissue engineering that require micron-scale particles such as the creation of macroporosity and delivery of cells.

FIG. 2. Schematic representation of the use of micro-/nanospheres for spatiotemporal control over delivery of bioactive molecules. **(A)** Spatial control of biomolecule delivery by employing a gradient distribution of micro-/nanospheres as delivery vehicles. **(B)** Temporal control of biomolecule delivery using micro-/nanospheres with differing release characteristics.

Strategies of Using Micro-/Nanospheres for Scaffolds Design

Generally, microspheres can be used as (i) a dispersed phase surrounded by a continuous matrix (solid polymers, hydrogel polymers, or CaP cements [CPCs]), or as building blocks to establish integral scaffolds without surrounding matrix by a bottom-up approach. In the next section, various strategies will be discussed that employ micro-/nanospheres for the design of scaffolds for bone tissue engineering with improved functionality.

Micro-/nanospheres as discrete components embedded into continuous matrices

By simply incorporating micro-/nanospheres into a continuous matrix (such as solid polymers, hydrogels, or CPCs), physicochemical and biological characteristics of these composite systems can be improved in terms of delivery of bioactive and/or chemical agents,¹²⁷ porosity,¹⁶ mechanical strength,¹⁷ and cell encapsulation.²²

Micro-/nanospheres embedded into solid polymers. One of the most common reasons to introduce micro-/nanospheres into solid (i.e., nonswelling) polymers is to provide bulk scaffolds with the capability of controlling the release of drugs.^{3–5,128,129} Especially for the delivery of bioactive molecules, the simple incorporation of growth factors into bulk scaffolds might lead to denaturation of these biomol-

ecules due to exposure to harsh preparation conditions, hydrophobic surfaces of polymers, acidic degradation products, and so on.¹³⁰ Previous studies have shown that incorporation of PLGA microspheres (as carriers for bone morphogenetic protein-2 [BMP-2]) into polyurethane scaffolds was accompanied by a reduced initial burst release followed by a sustained release of BMP-2 that promoted new bone formation compared with microsphere-free scaffolds.¹³¹

Moreover, the use of micro-/nanospheres as a delivery system allows for accurate spatiotemporal control over the release of growth factors, which are essential for successful bone regeneration by inducing osteogenesis as well as angiogenesis. To establish delivery of multiple biomolecules with programmed release kinetics, different micro-/nanosphere populations can be employed that carry various growth factors. By tailoring the physicochemical properties of these degradable spheres, distinct release behavior can be obtained, thus resulting in temporally controlled drug delivery¹³² (Fig. 2A). For example, dual delivery of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) was established by pre-encapsulating PDGF by using PLGA microspheres, which were subsequently incorporated into porous VEGF-containing PLGA scaffolds prepared by gas foaming technique.⁸³ Similarly, several other studies have focused on delivery of multiple growth factors by utilizing different microsphere populations to carry various biomolecules, such as the combination

FIG. 3. Experimental design and resulting photographs of two modes of cell delivery using hydrogels: cells were directly encapsulated into hydrogels to form conventional gel construct (top); alternatively, cell-laden microspheres were incorporated into hydrogels to form microsphere/hydrogel composite system (GC) (bottom).

The former strategy resulted in cell death, whereas the latter one led to cell survival and proliferation in the hydrogel. Cytoskeleton F-actin (red) was counterstained with nuclei (blue). Reprinted from 22 with permission. Copyright 2011, Elsevier.

of poly(4-vinyl pyridine) and alginate microspheres to load and release BMP-2 and BMP-7 independently.⁷⁴

Besides temporally controlled delivery, to spatially control the distribution of growth factors inside the scaffolds is also of substantial importance primarily owing to the concentration-dependent effect of growth factor.¹³³ Specifically for osteochondral tissue engineering, gradient-based bioactive signal delivery can induce both osteogenic and chondrogenic regeneration in the interfacial area.¹³⁴ To this end, Wang *et al.* developed scaffolds containing microspheres that formed growth factor gradients through the materials for osteochondral reconstruction.¹³⁵ In this study, BMP-2 and insulin-like growth factor I (IGF-I) (that induce osteogenic and chondrogenic differentiation of MSCs, respectively) were encapsulated by using silk or PLGA microspheres, which were subsequently embedded into porous silk scaffolds to form reverse concentration gradients of two factors (as illustrated in Fig. 2B). This signal gradients, in turn, stimulated human MSCs to differentiate into osteoblasts and chondrocytes, respectively.¹³⁵

Micro-/nanospheres embedded into hydrogels. Hydrogels made of biodegradable polymers are promising material candidates for regenerative medicine due to their unique combination of biocompatibility, biodegradability, and injectability.^{69,136,137} The incorporation of micro-/nanospheres into hydrogels can further upgrade the functionality of pure hydrogels from passively accepted implants to instructive and inductive scaffolds with improved physicochemical and biological properties.

First of all, the control over drug delivery kinetics is significantly improved on introduction of micro-/nanospheres into hydrogel matrices.¹²⁷ Additionally, micro-/nanospheres can serve as stimulus-sensitive delivery vehicles for biologically or chemically active agents to realize triggered release in response to external stimulation.³ A representative example of this latter approach was reported by Westhaus *et al.*, who incorporated spheres (liposomes) as functional components into alginate matrices to create stimulus-sensitive self-hardening injectables. Thermosensitive liposomes (which can be considered as nanospheres) loaded with Ca^{2+} ions were mixed with alginate solutions, and the gelation of the resulting mixture was subsequently induced by thermally triggered Ca^{2+} release from liposomes at 37°C.¹³⁸ These composite systems displayed excellent flowability at room temperature, while gelling rapidly at body temperature, which confirmed the potential of this composite system for use as injectable cell-laden or acellular bone defect fillers.¹³⁹ In a bioinspired approach toward tissue regeneration by using hydrogel matrices, micro-/nanosphere have also been considered as microscopic bioreactors that mimic matrix vesicles in human skeletal tissues. These vesicles act as microcapsules embedded in ECM to create a compartmentalized environment, for example, for the nucleation and formation of bone mineral.²⁰ By mimicking this natural process, calcium and phosphate-loaded liposomes were combined with collagen hydrogels, which induced the formation of apatite crystals and subsequent mineralization of hydrogels, thus forming self-hardening biomaterials for bone regeneration.¹⁹

Further, micro-/nanospheres can serve as reinforcement components¹⁷ or crosslinking agents¹⁸ to provide hydrogels

with additional mechanical support. For example, β -tricalcium phosphate (β -TCP) microsphere/alginate composite systems encapsulating MSCs have been developed as injectable 3D constructs for bone tissue engineering, in which inorganic microspheres of high stiffness reinforced the initial mechanical strength of the composites.¹⁷ Subsequently, the degradation of β -TCP provided sustained supply of Ca^{2+} as crosslinking agents to decelerate the degradation of the alginate matrix. On the other hand, spheres can also induce a physical crosslinking process into hydrogel-based constructs. For instance, positively charged PLA microspheres have been utilized to form a polyion complex with anionic polyelectrolytes (such as hyaluronic acid [HA]) to induce gelation of HA without introducing reactive chemicals that can be cytotoxic.¹⁸

Microspheres made of biodegradable and cytocompatible polymers can serve as a cell delivery vehicle to improve the biological performance of tissue engineering constructs. Conventional hydrogel-based cell delivery has shown limited success, primarily due to the lack of sufficient adhesion of anchorage-dependent cells (such as osteoblasts) to rather inert gels such as poly(ethylene glycol) (PEG)-based hydrogels. This phenomenon leads to cell death as well as the strict confinement of cells that impedes cell migration and cell-cell interactions.²² Therefore, the introduction of microspheres into hydrogels is an alternative way of providing focal adhesions and subsequent space for cell proliferation on concomitant sphere degradation.^{22,140–142} Wang *et al.* proposed an injectable osteogenic scaffold based on a cell-laden microsphere-encapsulated hydrogel using gelatin microspheres as cell carriers and agarose gels as a continuous matrix, which exhibited strong potential for cell transplantation and bone regeneration (Fig. 3).^{22,141,142}

Microspheres embedded into CPCs. Although CPCs have been extensively used for bone repair and regeneration, disadvantages still exist such as slow degradation rate *in vivo*, lack of macroporosity, and poor capability of controlled release. To improve their performance, biodegradable polymeric microspheres (made of e.g., PLGA,¹⁶ poly(trimethylene carbonate),¹⁴³ gelatin,^{144,145} and pectin¹⁴⁶) have been introduced into cements. Thus, macroporosity can be introduced on degradation of microspheres, which can create space for cell and tissue ingrowth and, subsequently, accelerate the resorption of CPCs.¹⁶ The incorporation of microspheres in CPC can further enable cell delivery into cements to form cell-laden cements that might speed up new bone formation.^{147,148} Xu and colleagues developed a hybrid system by encapsulating stem cells in alginate biodegradable microspheres that were subsequently incorporated into CPC, thus protecting cells from excessive fluctuations in pH and electrolyte concentrations during CPC setting reaction that are known to be detrimental for cell survival.^{147,148}

Micro-/nanospheres as building blocks for bottom-up fabrication of scaffolds

The traditional tissue engineering strategy typically employs “top-down” approach by loading a premade bulk scaffold with signaling biomolecules and cells to generate a cellularized construct that instructs toward bone tissue regeneration. This conventional approach is accompanied by

several drawbacks including limited cell penetration inside the scaffolds, loss of cell viability over time, poor control over drug delivery, and suboptimal clinical handling properties.^{149,150} On the contrary, the so-called “bottom-up” strategy toward developing scaffolds for tissue engineering has become increasingly attractive by developing novel engineered scaffolds with precise combination of cells, biomolecules, and synthetic biomaterials. In this approach, scaffolds of high functionality can be fabricated by an assembly of micro- or nanoscale particles as building blocks. To this end, micro- and nanospheres are obvious candidates as functional units, which can be equipped with desired physicochemical and biological properties to establish constructs that mimic the function and composition of natural tissues in the body. To engineer scaffolds by using particulate building blocks, three main fabrication strategies can be discerned, that is, synthesis by random packing, directed assembly, and rapid prototyping (RP) (as illustrated in Fig. 4).

Random packing. Micro-/nanospheres can be randomly packed together to form a macroscopic 3D scaffold¹⁵⁰ (Fig. 4A). This type of scaffold has been developed by using collagen,²³ gelatin,²⁴ fibrin,⁵⁹ chitosan,^{67,151} alginate,⁷⁸ and PLGA⁸⁶ micro-/nanospheres as building blocks for injectable formulations. By functionalizing each type of spheres separately, the structure of the resulting scaffolds can be precisely controlled at a microscale.¹⁵² For instance, the composition and function of the scaffolds can be customized by encapsulation of signaling biomolecules,¹⁵³ bioactive minerals,^{33,154} or by loading osteogenic cells at the outer sphere surface.

However, a critical concern of applying these microsphere-based scaffolds into bone defects is their poor integrity resulting from weak interparticle interactions, which lead to a poor mechanical stability c.q. high flowability of the scaffolds⁵⁴ and migration of individual particles from defect sites on implantation.¹⁵⁵ Therefore, different methods have been explored to preserve the agglomeration of micro-/nanosphere-based formulations at confined defect sites by using glues¹⁵⁵ or crosslinkers.¹⁵⁶ Another approach involves thermal fusion of polymeric microspheres into integrated macroscopic scaffolds as described by Laurencin's group by using PLGA¹⁵⁷ and chitosan¹⁵⁸ microspheres. The tight packing of microspheres resulted in porous scaffolds with high-pore interconnectivity, controllable pore size and amount of porosity, and mechanical properties comparable to cancellous bone.¹⁵⁷ Further studies on these sintered microsphere-based scaffolds confirmed their capacity to release biomolecules in a controlled manner,^{81,134} cytocompatibility *in vitro*,^{159,160} and osteoconductivity *in vivo*.¹⁶¹

Directed assembly (self-assembling scaffolds). Directed assembly of micro-/nanospheres into cohesive macroscopic constructs can be achieved by introducing attractive interparticle forces (such as electrostatic forces, magnetic forces, or hydrophobic interactions) (Fig. 4B). This approach overcomes the limitations of the random packing strategy and provides micro-/nanosphere-based scaffolds with enhanced structural integrity and mechanical stability. Due to the gentle physical crosslinking conditions that characterize these self-assembling systems, cytotoxic crosslinking chemicals to bridge particles are not necessary anymore. Additionally, irregular osseous defects can be filled

conveniently by using micro-/nanosphere-based formulations that exhibit excellent injectable and/or moldable as well as close packing of the spherical building blocks.

Assembly driven by electrostatic interactions. Charged micro-/nanospheres have been used for more than a decade as drug delivery vehicles, because polyion complexes can be formed with charged biomolecules due to attractive electrostatic interactions. Interestingly, electrostatic forces have also been found to serve as cohesive interparticle force to induce self-assembly of micro-/nanospheres of opposite charges.^{87,162} Consequently, colloidal gels based on dextran microspheres or PLGA nanospheres have been developed, which show great potential as scaffolds for bone tissue engineering. These gels exhibited excellent injectability, moldability, and capability of self-recovery after shearing, due to the formation of a physically crosslinked particulate network. In addition, these colloidal gels acted as reservoirs for sustained delivery of entrapped drugs with near zero-order drug release kinetic *in vitro* (for PLGA nanospheres¹⁶³) and stimulated osteoconductive bone formation *in vivo*.¹⁶³ However, challenges remained for these micro-/nanosphere colloidal gels, including (i) the necessity to derivatize dextran or PLGA by chemically grafting charged groups onto the polymer backbone,¹²⁵ (ii) the release of harmful degradation by-products of PLGA, (iii) the absence of cell-adhesive peptide sequences required for cell attachment, and (iv) the disruption of the network structure by screening of particle charges at a low pH or high ionic strength.¹⁶²

To overcome these disadvantages, oppositely charged gelatin nanospheres have been developed by using commercially available cationic and anionic gelatin, which facilitated the fabrication of gelatin micro-/nanospheres with positive and negative charges without the necessity of additional functionalization.⁵² As a result, the combination of oppositely charged gelatin nanospheres gave rise to injectable and biodegradable colloidal gels with high elasticity at low nanosphere concentrations owing to electrostatic self-assembly between and tight packing of gelatin nanospheres (Fig. 5). Due to their favorable clinical handling, ease of functionalization, and cost effectiveness, these gels show great potential for application as bone fillers for tissue regeneration and/or programmed drug release of multiple biomolecules at a predetermined release rate.

Assembly driven by magnetic interactions. In view of the increasing research interest in targeted drug delivery, the potential of magnetic micro-/nanospheres has also been extensively investigated. Magnetic spheres can be prepared by entrapping magnetic iron oxide nanoparticles within or onto the surface of micro-/nanospheres.¹⁶⁴ For instance, magnetic nanoparticles have been entrapped into dexamethasone-containing PLGA microspheres, which subsequently functioned as targeting drug carriers to provide localized and sustained drug release for the treatment of bone-related diseases.¹⁶⁵ These magnetic delivery vehicles increase the spatial accuracy and elongate drug action due to an increased residence time at the targeting site. Further, magnetic spheres have also been used to render commercially available scaffolds magnetic,¹⁶⁶ thus developing magnetic scaffolds that attract and uptake magnetic microcarriers loaded with bioactive agents via magnetic forces.

FIG. 4. Schematic representation of using micro-/nanospheres as building blocks for bottom-up design of scaffolds by random packing (A), directed assembly (B), and rapid prototyping (C). Directed assembly can be induced by interparticle forces such as electrostatic (D), magnetic (E), and hydrophobic (F) interactions.

Similar to charged particles, magnetic micro-/nanospheres can also be used as building blocks to induce self-assembly into macroscopic constructs. Alsberg *et al.* combined thrombin-coated magnetic microspheres with a fibrinogen solution to form fibrin gels with defined architecture at the nanoscale in which magnetic forces had been used to position thrombin-coated magnetic microspheres in a defined two-dimensional array to guide the self-assembly of fibrin fibrils.¹⁶⁷

Strikingly, magnetic nanospheres have also been utilized to pattern cells and form a scaffold-free cellularized structure for tissue engineering and regeneration. Magnetic cationic liposomes (MCLs) have been prepared that can be uptaken by cells via electrostatic attraction between cationic liposomes and negatively charged cell membranes.^{14,15} These liposome-labeled cells can be further guided by using a magnet to form complex cell patterns with 3D multilayered

FIG. 5. Schematic diagram (A) and resulting photographs (B–F) of injectable colloidal gels based on using oppositely charged gelatin nanospheres as building blocks showing the self-assembly (B, C) and gel formation (D–F) of gelatin nanospheres of opposite charge ("+" and "-" denote positive and negatively charged particles, respectively). Adapted from 54 with permission. Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

FIG. 6. Scaffolds consisting of calcium phosphates/poly(hydroxybutyrate-co-hydroxyvalerate) composite microspheres prepared by rapid prototyping using selective laser sintering technique. (A) Schematic diagram of the scaffold model designed by a computer; microcomputed tomography (B) and scanning electron microscopy (C, D) of the resulting scaffolds after rapid prototyping. Adapted from 170 with reproduction permission. Copyright 2011, Elsevier.

cellular structure.¹⁵ For example, MSCs were magnetically labeled with MCLs and cultured under the influence of a magnetic field, which induced the formation of a multi-layered structure after 24 h, whereas the MSCs maintained the ability to differentiate into various cells including osteoblasts after long-term *in vitro* cell culture.¹⁶⁸ Further *in vivo* studies revealed that these cellular constructs improved new bone formation, which confirmed the great potential of applying this scaffold-free methodology to bone tissue engineering.

Assembly driven by hydrophobic interactions. Self-assembling hydrogels based on oligolactate-grafted dextran microspheres have been developed by employing hydrophobic interactions between oligolactate chains on the surface of microspheres as the driven forces.¹⁶⁹ The resulting microscopic network displayed high elasticity with tailorable gel properties by modifying the chemical and physical composition of the microsphere-based gels. Interestingly, the gels showed self-recovery behavior after shear thinning, which indicated that the physical crosslinking of the gel network was reversible, which is beneficial for potential use as injectable formulation in tissue regeneration.¹⁶⁹

Rapid prototyping. RP techniques have been recently advocated for the design of bone tissue engineering scaffolds. Using RP, constructs with customized architecture can be created from computer-aided-design data using micro-/nanospheres as building blocks to assemble into scaffolds layer by layer (Fig. 4C). For instance, microspheres consisting of polymers (such as poly(hydroxybutyrate-co-hydroxyvalerate) [PHBV]) or composite (such as CaP/PHBV) have been prepared by using emulsification, which were further fabricated into 3D porous scaffolds by using

selective laser sintering (Fig. 6).^{170,171} The resulting scaffolds exhibited an interconnected porous structure and a high amount of porosity, thereby serving as a suitable environment for osteoblastic cell attachment, proliferation, and differentiation.

Besides the application of RP techniques for scaffold design, Mironov *et al.* have introduced the novel concept of 3D organ printing, in which scaffold-free cellularized tissues or organ constructs can be fabricated layer by layer by using tissue spheroids as building blocks.^{172,173} In continuous organ printing procedures, tissue spheroids can be dispensed with or without hydrogels as a carrier.¹⁷³ This new technology may represent a viable alternative to traditional approaches in tissue engineering based on biodegradable scaffolds.

Conclusions

The potential of microspheres in bone tissue engineering has been explored for several decades, whereas nanospheres have been increasingly advocated over the past decade. Generally, microspheres can be used as either a dispersed phase surrounded by a continuous matrix (solid polymers, hydrogel polymers, or CPCs) or as building blocks to establish integral macroscopic scaffolds by a bottom-up approach without surrounding matrix. Compared with traditional monolithic scaffolds, scaffolds for bone regeneration comprising micro- and nanospheres display advantages that are related to (i) an improved control over sustained delivery of therapeutic agents, signaling biomolecules, and even pluripotent stem cells, (ii) the introduction of spheres as stimulus-sensitive delivery vehicles for triggered release, (iii) the use of spheres to introduce porosity and/or improve the mechanical properties of bulk scaffolds by

acting as porogen or reinforcement phase, (iv) the use of spheres as compartmentalized microreactors for dedicated biochemical processes, (v) the use of spheres as a cell delivery vehicle, and, finally, (vi) the possibility of preparing injectable and/or moldable formulations to be applied by using minimally invasive surgery.

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Disclosure Statement

No competing financial interests exist.

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