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MICRO- AND NANOSPHERES FOR TISSUE ENGINEERING

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9.1 INTRODUCTION

The emergence of regenerative medicine has resulted in a novel interdisciplinary field that focuses on repair, replacement, and regeneration of diseased or damaged tissues or organs.[1–3] Despite extensive efforts in the past several decades, only limited success has been reported for synthetic biomaterials in the clinical setting, and autologous and allogeneic tissues are still widely accepted as the "gold standard" for tissue regeneration therapies. As one of the most important strategies in regenerative medicine, the field of tissue engineering (which typically combines biodegradable scaffolds, (stem) cells, and bioactive signals such as growth factors) has created new possibilities to produce implantable tissues *ex vivo*. After several decades of development, however, the simple combination of cells and biomaterials is still far from leading to successful tissue reconstruction. One limitation that restricts its widespread application is the basic design and preparation of conventional biomaterials, which generally fail to stimulate the human body's inherent capability of self-healing. Therefore, there is a strong demand for a new generation of

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biomaterials of enhanced complexity and functionality that not only provide architectural support for cell/tissue growth but also, more importantly, mimic the complex interactions between cells and the extracellular matrix (ECM) to orchestrate cellular behavior and induce functional tissue regeneration.

Micro- and nanospheres have drawn increasing interest in the field of regenerative medicine during the past decade, which can be used as functional components in novel biomaterials of improved functionality. Microspheres (here defined as ranging in diameter from 1 to 1000 μm) have been investigated for biomedical applications for several decades, but studies on the application of nanospheres (defined here as spheres with diameters between 10 and 1000 nm) in tissue engineering only emerged in the past 10 years, thus reflecting the rapid development of micro- and nanotechnology in the field of tissue engineering. With respect to the use of micro- and nanospheres for tissue engineering and regeneration, four major strategies can be discerned to introduce these spheres as functional components to improve the performance of conventional bulk biomaterials. First, micro- and nanospheres can be used for controlled delivery of therapeutics, chemical agents, and even cells; the spheres act as delivery vehicles because of their inherently small size and corresponding large specific surface area. The size and morphology of micro- and nanospheres facilitate a high drug-loading efficiency, a quick response to stimuli from the surrounding environment, a high reactivity toward surrounding tissues *in vivo*, and a high diffusibility and mobility of drug-loaded particles.[4–12] Specifically, by incorporating spheres loaded with biomolecules of interest into a continuous matrix, classical scaffolding biomaterials can release signaling molecules without compromising the properties of the bulk scaffold.[4–6,13] Second, micro- and nanospheres can be used to alter the mechanical performance of monolithic scaffolds either by acting as porogens to create porosity in otherwise dense scaffolds[14] or as reinforcement phase to improve the mechanical strength of weak matrices.[15,16] Third, by creating a protective microenvironment inside the spheres, micro- and nanospheres can be used as compartmentalized microscopic bioreactors for dedicated biochemical processes.[17] For instance, micro- and nanospheres can be used to induce formation of biominerals and subsequently trigger mineralization of surrounding hydrogels to form self-hardening biomaterials. This strategy is inspired by the process of endochondral bone formation, in which matrix vesicles function as microcapsules to create a compartmentalized environment for the nucleation and formation of bone mineral.[18] Fourth, micro- and nanospheres can serve as building blocks to establish macroscopic, shape-specific colloidal systems that can be used as injectable or moldable scaffolds for tissue engineering. This bottom-up strategy for design and manufacture of biomaterials has recently been advocated as a promising method to develop materials with a highly defined structure and precisely controlled properties.[19,20]

This chapter focuses on the most recent advances in research on micro- and nanospheres aiming at improvement of the functionality and clinical efficacy of traditional scaffolds for soft and hard tissue engineering.

9.2 MATERIALS CLASSIFICATION OF MICRO- AND NANOSPHERES

With regard to applications in tissue engineering, micro- and nanospheres should fulfill the basic requirements that apply to virtually all biomaterials, including biocompatibility, biodegradability, nontoxicity of degradation products, and ease of processing. In general, micro- and nanospheres can be categorized into polymeric, ceramic, and composite materials.

Polymeric micro- and nanospheres have been studied most extensively for applications in controlled delivery and tissue engineering since the 1970s, when polymeric microspheres were initially introduced as drug delivery systems. The advantages of polymers over inorganic biomaterials include the ease of processing, high degree of control over the physicochemical properties (such as biodegradability), and ease of functionalization and modification. Depending on their origin, polymeric micro- and nanospheres can be classified as either natural or synthetic polymers, both of which have their specific pros and cons.

Natural polymers are an important class of biomaterials in tissue regeneration basically because of their intrinsic biocompatibility and biodegradability. Because they are derived from natural organisms, natural polymers are generally characterized by an excellent biocompatibility, biodegradability, a negligible immunogenicity, an abundant presence of side groups allowing for further chemical functionalization, and the presence of cell-recognition motifs (in the case of protein-based polymers, e.g., collagen, gelatin and fibrin).[12,21–23] Micro- and nanospheres made of natural polymers can be prepared by simple emulsion techniques in which spheres of variable properties (size and morphology) can be obtained by tailoring the emulsification process.[24,25] The resultant micro- and nanospheres are widely accepted as desirable vehicles for drug or biomolecule delivery because of the gentle gelling conditions that facilitate encapsulation of biomolecules and cells, controllable release kinetics by fine-tuning the degradation of carriers, and ease of functionalization.[26–29] Despite the favorable properties, several critical concerns about natural polymers include (1) poor mechanical properties that hamper applications under load-bearing conditions,[21,22] (2) immunogenicity or the risk for disease transfer for polymers extracted from allogeneic or heterogenous sources,[30] and (3) poor control over physicochemical characteristics (e.g., molecular weight).

On the other hand, synthetic polymers, such as poly(lactic acid) (PLA) and poly (lactic-*co*-glycolic acid) (PLGA), are also of considerable importance for regenerative medicine applications owing to their biocompatibility, biodegradability, well-defined physicochemical properties (e.g., molecular weight), defined mechanical properties, ease of fabrication and modification, and the absence of the possibility to transfer diseases. Micro- and nanospheres composed of synthetic polymers have been widely investigated as delivery vehicles for therapeutic agents[31–33] and building blocks for tissue engineering scaffolds.[34,35] However, drawbacks related to the use of micro- and nanospheres made of synthetic polymers include the acidic degradation products, hydrophobicity, degradation by autocatalysis, and low drug-loading efficacy.[36]

To develop biomaterials of enhanced physicochemical and biological properties, composite materials have gained considerable attention for tissue engineering

applications over the past decades. By incorporating different components, composites combine the advantages but eliminate the drawbacks of each component, resulting in improved functionality and complexity. Representative examples include inorganic–organic composites for bone reconstruction, which typically combine biodegradable polymers with bioactive ceramics, resulting into materials that improve the biological performance of polymers as well as provide bioceramics with the ease of processing and controllable degradation. Composite micro- and nanospheres have been fabricated by incorporating bioceramics (e.g., calcium phosphates (CaPs)) with biopolymers (e.g., gelatin, PLGA),[37–44] which displayed improved biological and physicochemical properties that include enhanced hydrophilicity (compared with pure PLGA microspheres),[45] higher drug-loading efficiency,[46] improved cytocompatibility,[45] reduced biodegradation and drug release rates,[38] and strongly upregulated *in vitro* calcifying capability.[38]

9.3 APPLICATIONS OF MICRO- AND NANOSPHERES IN TISSUE ENGINEERING

9.3.1 Micro- and Nanospheres as Delivery Vehicles

9.3.1.1 Delivery of Biomolecules A critical challenge in tissue engineering is to control the delivery of signaling biomolecules at the treatment sites to provide instructive signals that regulate cell behavior and facilitate tissue regeneration. To this end, complex and sophisticated delivery systems are required that allow for sustained presence of therapeutic components at target tissues at the proper time. Micro- and nanospheres have been studied most extensively for controlled delivery of biomolecules owing to their inherently small size and corresponding large specific surface area, high drug-loading efficiency, high reactivity toward surrounding tissues *in vivo*, and high diffusibility and mobility of drug-loaded particles (Fig. 9.1).[4–8,13]

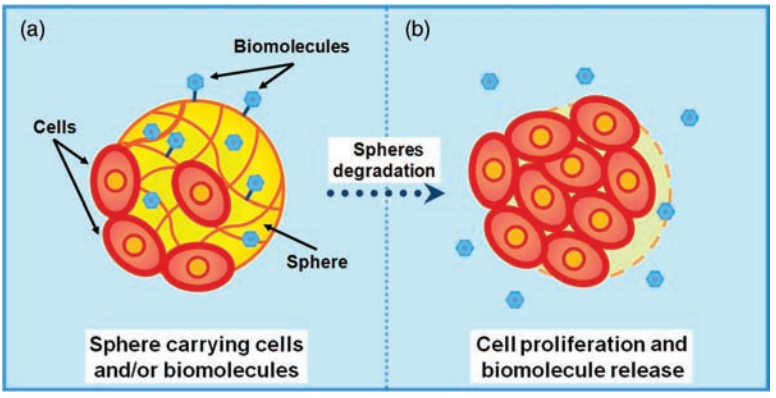

FIGURE 9.1 The use of degradable microspheres as vehicles for biomolecule or cell delivery (a), which can lead to subsequent cell proliferation and biomolecule release with the degradation of carriers (b).

The basic strategy to use micro- and nanospheres as carriers to load biomolecules is by simply adsorbing onto the particle surface and subsequently releasing their payload *in vivo* by desorption, diffusion, or carrier degradation depending on the chemical composition and geometry of the spherical vehicle. The poor control over the release of biomolecules is the main drawback that has limited widespread application of this method. Therefore, new methods have been developed that either (1) physically entrap biomolecules in the carrier matrix or (2) chemically immobilize biomolecules to the polymer backbone. Subsequently, release can be obtained upon degradation of the spheres or hydrolytic or enzymatic cleavage of the chemical bond between the carrier and the biomolecule.[47–49] More specifically, microencapsulation has been developed as a promising strategy for controlled delivery of biomolecules by physically compartmentalizing biomolecules into the hollow interior of micro- and nanospheres, thus protecting labile biomolecules from denaturation by harsh environmental factors. Release profiles of encapsulated biomolecules normally display sustained-release kinetics favorable for long-term delivery compared with molecules adsorbed onto surface of carriers.[50,51] Nkansah et al. prepared PLGA micro- and nanospheres with ciliary neurotrophic factor encapsulated inside the spheres by emulsification, which can be used as delivery vehicles for growth factor delivery without compromising their bioactivity.[52] Alternatively, biomolecules loaded into spherical carriers by chemical immobilization techniques normally show prolonged retention at the delivery site with a target-specific manner.[4,53–55]

Regarding the clinical application of drug-loaded micro- or nanospheres, one simple delivery strategy involves incorporation of micro- and nanospheres loaded with therapeutic components into a continuous matrix of monolithic scaffolds, thus prolonging the retention of biomolecules at the implantation site but also providing bulk scaffolds with enhanced features for controlled and sustained release of drug or proteins.[4–6,56–58] Especially for controlled delivery of biomolecules, simple incorporation of biomolecules into bulk materials probably leads to their denaturation or deactivation caused by exposure to harsh processing conditions, hydrophobic surfaces of polymers, or acidic degradation products.[58] However, incorporating biomolecule-loaded spheres into polymer scaffolds was found to be of more efficiency with a reduced initial burst release followed by a slow, sustained release of biomolecules compared with a release profile using microsphere-free scaffolds.[59]

Moreover, programmed delivery of multiple biomolecules with precise spatiotemporal control over the distribution of biomolecules throughout scaffolding materials or sequential release of various molecules can be achieved by incorporating micro- and nanospheres as delivery system into classical scaffolds.[33,60] Temporal control over biomolecule delivery can be realized by using various microsphere or nanosphere populations for different biomolecules. In doing so, distinct release profiles of each components can be obtained by tailoring the physicochemical properties of each spheres and the corresponding release behavior, resulting in temporally controlled drug delivery.[60] For example, sequential release of dual growth factors was obtained by combining both poly(4-vinylpyridine) and alginate microspheres to load and release bone morphogenetic protein 2 (BMP-2) and BMP-7 independently.[61] Furthermore, spatial control of signaling molecules is of growing

interest for engineering of many tissues such as nervous[62] and osteochondral[63] tissues. In these applications, a gradient distribution of bioactive signals is established to induce concentration-dependent cell responses.[64,65] To this end, Wang et al. developed scaffolds containing reverse concentration gradients of two growth factors (BMP-2 and insulin-like growth factor I (IGF-I)) through polymer scaffolds for osteochondral reconstruction by introducing silk and PLGA microspheres as carriers for each growth factor.[66] In that way, human mesenchymal stem cells (MSCs) were stimulated to differentiate into osteoblasts and chondrocytes, respectively.

9.3.1.2 Delivery of Cells Besides delivery of therapeutic or biochemical components, biodegradable and cytocompatible microspheres can also serve as cell delivery vehicles to improve the biological performance of tissue engineering constructs (Fig. 9.1) or to construct microscopic three-dimensional (3D) tissue equivalents that mimic the native tissue structure. In contrast to conventional hydrogel-based cell encapsulation approaches that normally lead to cell death because of limited cell adhesion, migration, and communication,[67] the introduction of microspheres as cell carriers into hydrogels not only provides cellular focal adhesions (in case of arginine-glycine-aspartic acid (RGD)-containing polymers) but also facilitates cells to overcome gel restriction and fully spread out into their natural morphology.[67–70] Wang et al. proposed an injectable hydrogel scaffold based on encapsulation of cell-laden gelatin microspheres into a continuous matrix of agarose hydrogel, which exhibited strong potential for cell conveyance and regeneration of bone and other tissues.[67,69,70] Considering the above mentioned approach as traditional scaffold-based "top-down" strategy to create cellularized constructs, "bottom-up" tissue fabrication methods using cell-laden microspheres as building blocks are potentially more powerful tools to construct 3D hybrid constructs comprising both cells and biomaterials.[71,72] Matsunaga et al. recently developed a method for rapid construction of macroscopic 3D constructs using a large number of monodisperse cell-laden collagen microspheres with monodispersity to assemble into uniform and shape-specific tissues.[71] Similarly, Pautot et al. proposed a colloidal superstructure based on monodisperse silica microspheres for 3D neuronal network formation. These microsphere-based bottom-up strategies showed many advantages, including (1) a large surface area provided by microspheres for cell adhesion and further functionalization; (2) abundant interparticle cavities, allowing for nutrient exchange *in vitro* and *in vivo*; and (3) ease of manipulation and transportation of colloidal microspheres.[71,73]

9.3.2 Micro- and Nanospheres as Functional Components to Modify Mechanical Properties of Scaffolds

9.3.2.1 Use of Micro- and Nanospheres as Porogens By embedding microspheres into the continuous matrix of bulk materials, spheres can serve as porogen to introduce porosity into otherwise dense biomaterials (Fig. 9.2). A typical example of this strategy is the incorporation of microspheres into calcium phosphate cements (CPCs), which exhibit slow degradation rates *in vivo* and consequently a lack of

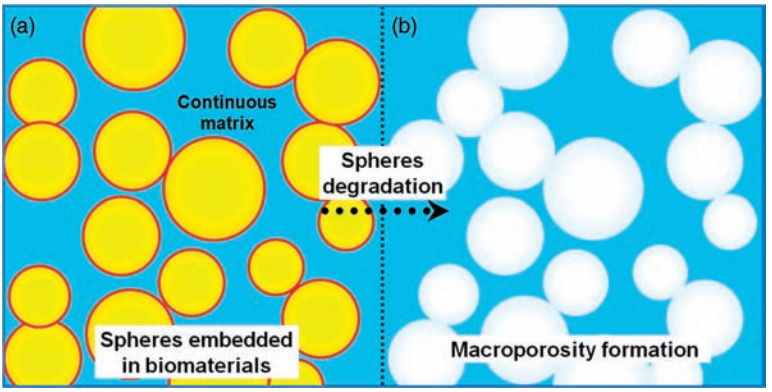

FIGURE 9.2 The use of spheres as porogens to introduce macroporosity to otherwise dense biomaterials by embedding spheres into the continuous matrix of bulk materials (a), thus creating porosity with the degradation of the spheres (b).

macroporosity and new bone ingrowth. By introducing degradable polymeric microspheres (made of, e.g., PLGA[14] and gelatin[74,75]), macroporosity can be formed introduced upon degradation of microspheres, which can subsequently create space for cell and tissue ingrowth and accelerate the resorption of CPCs.[14] Additionally, this strategy can make injectable CPCs suitable for cell encapsulation and biomolecule delivery to upregulate the extend of bone regeneration even further.[76,77]

Another method to use micro- and nanospheres as porogens to create porous structures is a technique referred to as *colloidal crystal templating*. This technique involves the formation of a body of closely packed monodisperse spheres, after which the interstitial space is filled with a solidifying fluid precursor followed by removal of the template to obtain a porous inverse replica.[78] The resultant so-called inverted colloidal crystal (ICC) scaffolds with highly ordered macroporosity displayed significant advantages compared with traditional processing techniques, including a tightly controlled pore size (ranging from nanometer to micrometer scale); a well-defined periodic hierarchical porous structure with high interconnectivity; a highly accessible surface and large pore size; and the possibility to include pores of different sizes, allowing for selective uptake of small or large biomolecules.[79–81] Moreover, scaffolds produced using this technique are characterized by a uniform distribution of cells throughout the porous matrix, thus creating a highly standardized microenvironment for cell encapsulation.[82]

9.3.2.2 Use of Micro- and Nanospheres as Reinforcement Components Micro- and nanospheres can be incorporated into continuous matrices to provide additional mechanical support for traditional biomaterials by serving as reinforcement components[15] or cross-linking agents.[16] Ceramic micro- or nanoparticles are favorable candidates in the reinforcement phase to be incorporated into polymer matrices because of their intrinsically higher mechanical strength. For instance, β-tricalcium phosphate (β-TCP) MSCs were combined with alginate hydrogels to form an

injectable 3D constructs that can encapsulate MSCs in which β-TCP microspheres of high stiffness reinforced the mechanical strength of the alginate matrix.[15] On the other hand, micro- and nanospheres embedded into the continuous phase of biomaterials can also act as cross-linking anchors to form direct bridges between micro- and nanospheres with the surrounding network or function as delivery vehicles that encapsulate cross-linking agents and subsequently release them to trigger cross-linking of the surrounding polymer phase. For instance, positively charged PLA microspheres were embedded in an anionic polymer phase of hyaluronic acid to induce gelation of hyaluronic acid by forming polyion complexes without introducing cross-linking chemicals that can be cytotoxic.[16] Moreover, in the design of so-called self-healing biomaterials, microspheres can be used as microcapsules containing an active healing agent dispersed in a polymer matrix. When a propagating crack encounters a microcapsule and causes its rupture, the healing agent is released to initiate a repolymerization process, thus filling the crack area.[83] This approach of using microspheres in designing self-healing biomaterials is an excitingly new area that can be of great benefit in the development of novel biomaterials.

9.3.3 Micro- and Nanospheres as Microreactors

Hollow micro- and nanospheres (microcapsules) have been investigated recently for their potential to serve as microscopic bioreactors for dedicated biochemical processes in biomedical applications.[17,84,85] Candidates for this purpose include polymeric capsules, liposomes, polymersomes, and so on that can (1) create a inner compartment capable of efficient entrapment of components of interest; (2) provide a sufficiently robust and stable shell, allowing for selective diffusion of substrate components or reaction products into or out of the capsules; and (3) introduce no harmful effect to native cells and tissues.[85,86]

A representative example of using microcapsules for biomedical applications is the controlled formation of biominerals in defined compartments. This strategy is inspired by the process of endochondral bone formation that uses nanosized matrix vesicles as initial sites of biomineralization.[17,18] To this end, Michel et al. developed an approach using liposomes encapsulated with calcium ions and alkaline phosphatase (the enzyme that releases inorganic phosphate ions from organic phosphate esters *in vivo*) to induce CaP crystals formation under well-controlled conditions (Fig. 9.3).[87] Similarly, Pederson et al. developed calcium- and phosphate-loaded liposomes in combination with collagen hydrogels, which facilitated *in situ* formation of CaP crystals and subsequent mineralization of hydrogels, and finally formed self-hardening biomaterials that can be applied as injectable, self-gelling formulations for bone regeneration.[17] Another biomimetic approach for inducing biomineral formation inside polyelectrolyte capsules was developed by Antipov et al.[88] based on urease-catalyzed precipitation of carbonate in the capsule interior. By suspending urease-loaded capsules in aqueous solutions containing $CaCl_2$ and urea, $CaCO_3$ mineralization was triggered because of the impermeability of urease macromolecules inside the capsules, but high permeability of small urea molecules and Ca^{2+} through the capsule wall allowed for precipitation of inorganic crystals. These

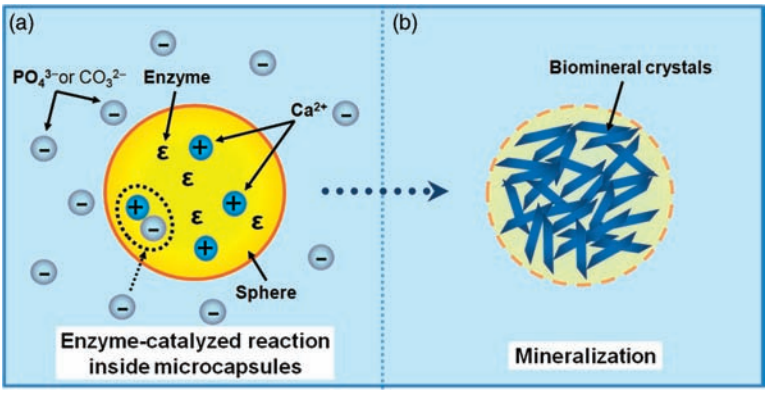

FIGURE 9.3 The use of spheres as microscopic bioreactors for controlled formation of biominerals. Enzyme-catalyzed reaction can be triggered in a defined compartment (a), thus facilitating the nucleation and formation of biominerals crystals inside microcapsules (b).

dedicated biomimetic strategies for biomineral formation using defined microcapsules provide a promising pathway for the development of hybrid organic–inorganic biomaterials that can be used for tissue engineering and regeneration.

9.3.4 Micro- and Nanospheres as Building Blocks

Recently, "bottom-up" strategies for the design of novel biomaterials have been advocated as a new paradigm for development of a new generation of tissue engineering scaffolds. One example of such a bottom-up strategy uses micro- or nanoscale particles as building blocks to (self-)assemble into macroscopic structures. Micro- and nanospheres are obvious candidates as structural building blocks for such applications, in which integrated structures can be formed by either random packing or directed self-assembly (Fig. 9.4). As opposed to traditional monolithic implants, these sphere-based scaffolds display several advantages for tissue engineering such as a precise control over the physicochemical characteristics of scaffolds (e.g., degradation rate) by fine-tuning the specific structural units,[89] the ease of encapsulation of therapeutic[90] or biochemical[29,91] components, and desirable clinical handling properties (i.e., injectability and moldability).[72,92]

The most basic strategy to create scaffolds composed of micro- and nanospheres is by randomly packing the spherical building blocks together, which normally results in a moderately organized 3D structure of poor cohesion.[72] Polymeric micro- and nanospheres (e.g., gelatin,[20] chitosan,[93,94] alginate,[95] and PLGA[34] micro- and nanospheres) have been used to build up scaffolds by simply packing them together, thus forming injectable formulations that can be used as defect fillers for tissue regeneration. However, one critical concern of this strategy for *in vivo* applications is the poor integrity of the spheres because of the lack of interparticle interactions, which could potentially lead to poor mechanical stability and high flowability of the scaffolds[96] and ultimately detrimental side effects to the surrounding tissues caused

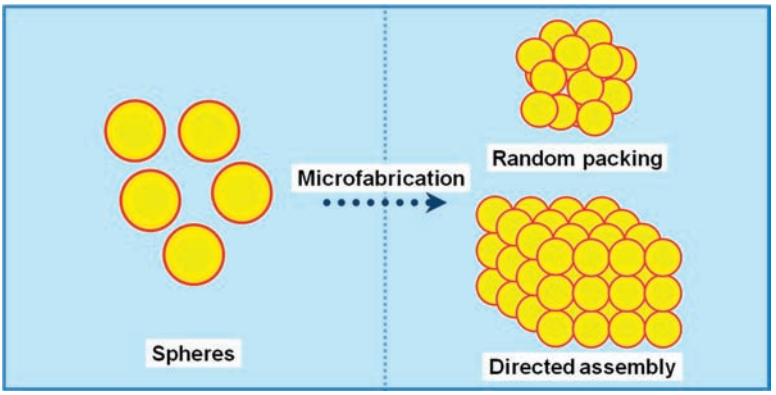

FIGURE 9.4 The use of micro- and nanospheres as building blocks to assemble into macroscopic structures by either random packing or directed assembly of the spheres.

by the individual particles migrating from the treatment sites.[97] To address this problem, efforts have been made to increase the cohesion of micro- and nanosphere-based formulations at the implantation sites (e.g., by using glues or additional interparticle cross-linkers).[97,98] Alternatively, sintered microsphere-based scaffolds were developed by fusing densely packed PLGA[99] or chitosan[100] microspheres together by thermal treatments. These scaffolds exhibited tailorable morphological and compositional properties of the scaffolds,[99] controllable biomolecules release profiles,[31,63] *in vitro* and *in vivo* biocompatibility,[101,102] and a degree of degradability suitable for tissue engineering applications.[103]

Directed assembly of micro- and nanospheres into cohesive macroscopic constructs has recently been advocated as a more sophisticated strategy to design particle-based scaffolds by maximizing interparticle interactions (e.g., electrostatic, magnetic, or hydrophobic interactions) as driving forces to induce self-assembly of micro- and nanospheres. Specifically, colloidal gels have been developed recently based on self-assembly of micro- or nanospheres directed by either electrostatic[35,104–106] or hydrophobic[107] interactions, which showed desired structural integrity and mechanical stability in physiological conditions,[105] excellent injectability and moldability, and capability of self-recovery after network destruction because of the reversible physical cross-linking features that characterize these self-assembling systems.[35,105,107] These physical gels showed great potential to be used as injectable fillers for regenerative medicine by using minimally invasive surgery. For example, Wang et al. prepared injectable colloidal gels made of oppositely charged, dexamethasone-loaded PLGA nanospheres, which displayed a nearly zero-order drug release profile *in vitro* and induced bone formation *in vivo*.[108] Similar to electrostatic and hydrophobic interactions, magnetic force can also be used as a powerful tool to trigger self-assembly of micro- or nanoscale building blocks to generate integrated structures as tissue engineering scaffolds.[109,110] Interestingly, instead of using magnetic micro- and nanospheres as building blocks, Ito et al. recently developed magnetic nanosphere-labeled cells as structural units to form a

scaffold-free, cell-patterned structure.[111–113] This so-called magnetic force-based tissue engineering strategy showed potential to construct 3D cellularized tissues even without using monolithic scaffolds.

9.4 CONCLUSIONS

Micro- and nanospheres have evolved as powerful tools in the design of novel biomaterials for controlled delivery and tissue engineering and regeneration. Strategies using micro- and nanospheres display several advantages compared with conventional monolithic biomaterials, such as (1) an improved performance in controlled and sustained delivery of therapeutic agents, signaling biomolecules, and even (stem) cells; (2) improved structural or mechanical properties of bulk scaffold by using spheres as porogens or reinforcement phase to introduce porosity or improve mechanical strength; (3) upregulated control over dedicated biochemical processes by using micro- and nanospheres as compartmentalized microreactors; and (4) the possibility to prepare self-assembling colloidal systems that can be used as injectable or moldable formulations to be applied using minimally invasive surgery.

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