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INTEGRATING TOP-DOWN AND BOTTOM-UP SCAFFOLDING TISSUE ENGINEERING APPROACH FOR BONE REGENERATION

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6.1 INTRODUCTION

Tissue engineering (TE) as an interdisciplinary field of research aims at restoring, maintaining, or improving tissue function through applying the principles of biology, medicine, and engineering science.[1] Since its emergence in the 1980s, the field of TE in conjunction with regenerative medicine has been continuing to evolve, for example, through wound healing,[2,3] skin tissue engineering,[4–6] nerve regeneration,[7,8] cardiovascular tissue engineering,[9] bone and cartilage tissue engineering,[10] and others.[11,12]

Cells, scaffolds, and growth-stimulating bioactive factors are generally referred to as the three key components of engineered tissues in TE.[1] A common strategy in TE is combining cells, biodegradable scaffolds, and bioactive factors to replicate natural processes of tissue regeneration and development.[10] The interactions among these components are imperative to achieve biologically functional engineered tissue. In human tissue, cells are normally anchorage dependent, residing in an extracellular matrix (ECM). This ECM generally provides not only structural support and a physical environment but also bioactive cues and a reservoir of growth factors.[13] The

Micro and Nanotechnologies in Engineering Stem Cells and Tissues, First Edition. Edited by Murugan Ramalingam, Esmaiel Jabbari, Seeram Ramakrishna, and Ali Khademhosseini.

synthetic scaffolds for an engineered tissue are regarded as a kind of ECM. However, ECM in native tissues possesses complex compositions and a dynamic nature, which bring multiple biological functions such as cell adhesion, migration, proliferation, and differentiation. Ideal scaffolds should therefore mimic the features of the native ECM of the target tissue. Nevertheless, the complexity of ECM makes it difficult to mimic exactly the structure and functions of native ECM in synthetic scaffolds.[14]

Therefore, the focus in tissue engineering is how to manipulate the process to integrate the key components of TE, trying to replicate the natural structure of tissue and mimic the functions of native ECM, at least partially. There are many technologies developed to achieve these aims. Although these techniques have succeeded in making biomimetic scaffolds, they have their own limitations. This chapter reviews the bone TE strategies involved in preparation of scaffolds and briefly discusses the drawbacks and advantages of these strategies.

6.2 CLINIC NEEDS IN BONE REGENERATION FIELDS

Every year, there are roughly 1 million bone grafting procedures in the United States and European Union.[15] These include indications arising from resection of primary and metastatic tumors, bone loss after skeletal trauma, failed fracture healing, spinal arthrodesis, and trabecular voids. In addition, more than 20 million people in the United States are totally edentulous.[16] About half a million children worldwide are born annually with congenital craniofacial deformities, such as cleft palate and hypertelorism.[17] Current treatments in clinic are based on autologous and allogeneic bone grafts.[18–22] Autografts have been the gold standard of bone replacement for many years because they provide the patient's own osteogenic cells, ECM, and essential osteoinductive factors needed for bone healing and regeneration.[21,23] Because an autograft is harvested from the patient's own body, there is a limited supply and morbidity of the harvest site, and the additional trauma is a concern. Although autograft is highly efficient for bone repair, the outcome for large bone defects is less predictable. Allografts could be used as an alternative for treating bone defects. However, allografts could introduce the possibilities of immune system rejection, pathogen disease transmission from donor to recipient, and infections after the transplantation.[24]

Therefore, biomaterials for bone defects, as an alternative to those two bone grafts, have been extensively studied to meet the increasing clinical demand. Currently, all kinds of biomaterials, including metals, ceramics, and polymers, have been studied for bone regeneration. However, none of these biomaterials, by themselves, can currently be used for full recovery of the patient. Metals exhibit poor integration with the tissue at the implantation site because of a lack of degradability, although they provide mechanical support at the site of the defect. Ceramics, because of their low tensile strength and brittleness, have limited application in loading-bearing sites. Polymers have been extensively used in drug delivery systems but have limitations in bone tissue engineering because of their low compressive strength and acid degradation products. It is clear that an adequate bone graft is yet to be found.

6.3 BONE REGENERATION STRATEGIES AND TECHNIQUES

Scaffolds need to mimic the natural structure of regenerated tissue to obtain optimal regeneration of biological functions. From a perspective of tissue engineering, cells, ECM, cell–matrix interactions, and bioactive factors should be involved to achieve the regenerated functions. For the components mentioned, an appropriate three-dimensional (3D) scaffold is an essential component for a tissue engineering strategy because scaffolds provide physical and mechanical support, spatial structure, and an adequate biochemical environment for cell behavior.[10] Scaffolds applied in TE need essential properties, including pore size, porosity, mechanical properties, and signal presentation.

Bone is a dynamic, highly vascularized tissue with hierarchic structure in a 3D configuration.[15] Therefore, the ideal scaffold should mimic the bone structure and provide a 3D microenvironment for growing new tissue in the scaffold. However, the coordination of all of these key components in an optimal spatial and time-dependent fashion will affect the ultimate results of regenerated tissues. There are many strategies or techniques for making bone constructs for tissue regeneration. From a fabrication perspective, these strategies can be generally implemented in two approaches: top down and bottom up.

6.3.1 Top-Down Tissue Engineering

6.3.1.1 Concept Since its emergence in the 1980s,[1,25] TE began to develop different approaches for tissue regeneration. The top-down approach represents the most traditional and typical one. Top-down tissue engineering approach generally uses well-defined porous scaffolds with tailored properties and signals as a template to induce desired cell response, leading to engineered tissues and organs. Specifically, to construct engineered bones, bone-forming cells or stem cells are seeded onto prefabricated porous scaffolds with controlled release of growth factors to induce bone formation. The essential properties of the scaffold include porosity, interconnectivity and pore size, mechanical strength, and biodegradability. Scaffolds as a template should possess similar functions to natural ECM. Scaffolds must possess a fully interconnected porous structure and open macropores for efficient nutrient and metabolite transport. The pores also facilitate the neovascularization of the construct from the surrounding tissue at the same time. However, the porosity will affect the mechanical properties that are required to balance the degradability of the scaffold. The mechanical properties of the implanted scaffold should ideally match those of living bone, so that no stress shielding or compression or deformation of the scaffold by the surrounding tissues takes place.[26–28] Therefore, the extent of porosity should be balanced with mechanical properties so that they both meet the demands of a specific regenerating tissue. To further enhance cellular adhesion and proliferation on the scaffold, the surface could be modified to be osteoconductive. Many different cell-interacting ligands, such as the RGD cell-adhesive ligand, could be grafted to the scaffold to provide biological cues for cell growth. The scaffolds may be used to load growth factors or to serve as a delivery vehicle or reservoir for

exogenous bioactive molecules to enhance regeneration. Many methods have been developed to produce scaffolds with adequate properties as mentioned earlier based on the top-down concept. An adequate processing technique should be performed on selected biodegradable materials. A description and discussion of these techniques is given in the following sections.

6.3.1.2 Processing Techniques Many techniques have been developed to prepare porous ceramic, polymer, or composite scaffolds. Gel casting of foams is an early developed technique for fabricating ceramic scaffolds with high mechanical strength.[29–31] This technique commonly results in a poorly interconnected pore structure and nonuniform pore size distribution;[32] however, these properties can be improved by using a sol–gel material and a gel-casting hybrid process.[33] The ceramic foam fabricated with this hybrid method exhibited sponge-like structures with uniform large pores and smaller pores distributed on the walls of the larger pores. The sizes of big and small pores were within 500–800 and 50–300 μm, respectively.[33]

Replication of a polymer sponge is a typical technique for producing ceramic scaffolds.[34,35] The replication method uses a sacrificial template (e.g., polyurethane foam) coated by a ceramic (or glass) slurry. After drying the ceramic slurry, the polymer template is slowly removed by thermal degradation, and the remaining ceramic is further sintered. The process replicates the macrostructure of the starting sacrificial polymer foam.[36–38] However, the low compressive strength of the scaffolds produced by this method limits their application in the repair of load-bearing bone defects.[39] Ramay and Zhang combined the gel-casting and polymer sponge methods to produce porous hydroxyapatite scaffolds with high mechanical properties.[32] A compressive modulus of 8 GPa and yield strength of 5 MPa for the scaffold with hydroxyapatite (HA) concentration of 50 wt% were achieved.[39] Fu et al. used a new method of direct-ink-write assembly of a hydrogel-based ink to fabricate bioactive glass scaffolds. Porous glass scaffolds with combined high compressive strength (136 MPa) and porosity (60%) were obtained,[40,41] which were comparable in mechanical properties to those of cortical bone and a porosity comparable to that of trabecular bone. The template-casting method is another technique that is used to produce porous ceramic scaffolds[41] and polymer scaffolds.[42–44] Recently, Yang and coworkers developed a template-casting technique to produce scaffolds with improved porous structure and mechanical strength. Scaffold composition and architecture were spatially regulated by controlling bead size and arrangement.[45–47]

For producing porous polymer scaffold, solvent casting and particulate leaching is the best known and most widely used method for the preparation of bone tissue engineering scaffolds because of its simple operation and adequate control of the pore size and porosity. After casting a dissolved polymer with a porogen, the solidified polymer is placed in a water bath to leach out the porogen, thus yielding an interconnected porous network. In this method, the particle size and amount of the porogen can be controlled. However, this technique is not applicable to ceramic scaffolds because the ceramic matrix obstructs complete removal of the porogen in the leaching step, resulting in a less interconnected network. Ever since Mikos et al. developed this technique to produce PLLA and PLGA polymer scaffolds,[48] many

researchers have used this technology to produce porous polymeric scaffolds.[49–53] There are many variations to the solvent casting and particulate leaching technique.[54] For example, any water-soluble porogen, different combinations of polymers and solvents, and varying compositions can be used in the casting step. The porogen can be also poured into a mold and partially fused using humidity to increase pore interconnectivity. PLLA and PLGA porous scaffolds have been produced with this modified method.[55–57] However, this method has some disadvantages; for example, the use of highly toxic solvents for polymer dissolution and the residual solvent remaining in the scaffold is a concern, and the residual porogen remaining in the polymer matrix after the leaching step can lead to enclosed and unconnected cavities.

In the thermally induced phase separation technique, a polymer such as PLLA, PLGA, or PCL is dissolved in an appropriate solvent (e.g., chloroform, dichloromethane) to obtain a homogeneous mixture. Next, the mixture is cooled below the solvent melting point to induce phase separation.[58] Then the mixture is quenched to form a two-phase solid, and the solvent is sublimated to yield a porous scaffold. The porosity and architecture of the polymer scaffold in this processing technique are generally affected by the cooling rate and melting temperature of the solvent.[59,60]

Freeze drying can also be used to fabricate scaffolds. An emulsified polymer solution is poured into a metal mold with the desired dimensions and allowed to freeze. Then the solvent is removed by freeze drying to yield a porous scaffold. However, the pores generated by this technique are relatively small.

Major concerns with typical solvent-casting strategies are the use of organic solvents and the toxicity of the residual solvent remaining in the scaffold after drying. A modified method is the gas-foaming technique, which does not require the addition of organic solvents. Compressed polymer disks in a mold are treated with high-pressure CO_2 or supercritical CO_2.[61–65] The nucleation in the polymer occurs when the pressure quickly decreases, thus forming pores. The pore size can be controlled by the reduction rate of pressure, but the pores produced by this technique are not interconnected. The combination of particulate leaching and gas foaming can improve the interconnectivity of the pores.[51,66]

Fiber bonding and electrospinning are fiber-fabricating technologies that create porous scaffolds composed of nano- and microscale biodegradable fibers. Many biocompatible polymers, such as PGA, PLGA, and PCL, are electrospun into porous nanofiber scaffolds with high porosities.[52,67]

Rapid prototyping by solid free-form technology (SFF) is used to produce porous scaffolds with well-defined pore geometry. This technology includes 3D printing,[68–70] laser sintering,[71–74] and stereolithography with variants.[75,76] Using computer-assisted design (CAD), this technique can produce fully interconnected porous scaffolds with well-defined pore geometry and complex pore architectures at the microscale. This technique has advantage over conventional fabrication techniques because the scaffold pore size and geometry can be designed electronically and mathematically. In a variant of this technique, a sacrificial wax mold is fabricated by an SFF technique such as fused deposition modeling (FDM). Then an *in situ* cross-linkable macromer is injected in the pore volume of the scaffold and allowed to cross-link by photo- or redox-initiated polymerization, rendering the polymer

insoluble in organic solvents. Then the infused mold is ether or wax, good solvent for ether, to leach out the wax, leaving behind a scaffold with well-defined pore geometry.[77] This technique can be used to fabricate porous uncross-linked or cross-linked polymer and hydrogel scaffolds with well-defined pore geometry. SFF technology has also been used to fabricate β-tricalcium phosphate (β-TCP)[78] and HA[79–81] scaffolds for bone regeneration. Toughness and strength in SFF scaffolds can be enhanced by adding a ceramic ink to the polymer phase.[82–85]

Other techniques for scaffold fabrication exist, such as melt molding and extrusion, which are not described here. These usually involve semi-industrial macrofabrication processes and extreme fabrication conditions, which are not compatible with the microscale environments for cells. However, the end product can be modified chemically after fabrication for cellular biocompatibility, although this can be more easily achieved by the previously described methods.

6.3.1.3 Limitations and Challenges The top-down approach using prefabricated scaffolds has a number of advantages. The materials used are diverse, ranging from ‘ceramics to polymers and hydrogels. These techniques can also produce porous scaffolds with high mechanical properties by altering the porosity and pore architecture. However, the top-down approach also has certain disadvantages. In this approach, the scaffold is expected to promote proliferation and differentiation of the cells seeded in the prefabricated biodegradable scaffold and create ECM. Although ceramic, polymer, and composite scaffolds fabricated by the top-down approach have been used as TE scaffolds, these porous biodegradable constructs often lack biological recognition cues. For example, they often lack osteoinductivity for bone tissue engineering. Postfabrication cell seeding into porous scaffolds is also inefficient because the ability of cells to penetrate the central part of the scaffold is limited, which leads to inhomogeneous distribution of cells in the scaffold and insufficient vasculature ingrowth. An ideal TE scaffold should mimic the native ECM and promote cell adhesion, growth, and differentiation.[86,87] To achieve this purpose and overcome drawbacks of the top-down approach, bioactive molecules, including growth factors, short peptides, and ECM proteins, are deposited, attached, or conjugated to the scaffold. For example, Jabbari and colleagues have shown that attached of a cell-adhesive RGD peptide and an osteoinductive peptide derived from bone morphogenetic protein 2 (BMP-2) synergistically enhances osteogenic differentiation of bone marrow stromal cells (bMSCs) and mineralized matrix formation.[88] Other efforts made to improve cell seeding include flow perfusion of the cell suspension inside the scaffold and using scaffolds with larger pore size.[89–91] However, despite these advances in surface engineering, biomimetic design, and conjugation methodologies to modify the scaffold microenvironment, top-down approaches still have difficulty recreating the intricate structure characters of tissues at micro- or nanoscale.

6.3.2 Modular Tissue Engineering (Bottom-Up Approach)

6.3.2.1 Concept The bottom-up approach aims to address the challenges of the top-down approach in mimicking the microstructural features of the tissue from the

opposite direction. The bottom-up approach builds a single unit at the micro- or nanoscale that serves as a building block for further assembly to a larger tissue scale. These modular units can be created in many different ways, such as cell sheeting, cell-laden microfabrication, or 3D direct cell printing. Then these units can be assembled to a larger tissue size by self-assembly or layering of cell sheets[92] to mimic the native microstructural repeating functional unit of the bone tissue. Bottom-up TE creates a more biomimetic engineered matrix at tissue level than the top-down approach.

6.3.2.2 Processing Techniques Micromolding and photolithography can be used to generate 3D cell-laden hydrogels. Micromolding of hydrogels provides a potentially powerful method for fabricating micro- and nanostructures.[93–95] Micromolding uses poly(dimethyl siloxane) (PDMS) molds microfabricated into a variety of shapes and sizes. In the first step, the prepolymer solution with the cell suspension is molded with PDMS mold. Then the solution is cross-linked by changing pH, temperature, ionic strength, or photoinitiator to generate a hydrogel with exact microstructures with the size and shape of the PDMS mold.[96] Many types of natural and synthetic hydrogels can be used for encapsulation of living cells, such as agarose,[97] chitosan,[98] and poly(ethylene glycol).[99] Collagen is a natural biocompatible and biodegradable material and has been extensively used to simulate the native ECM in tissues.

Photolithography provides another reliable technique to make microstructural modules with definite shapes, typically using photomasks with diverse patterns for patterning multiple cells in specific regions. Using this technology, a prepolymer solution of a cross-linkable hydrogel with photoinitiator is placed under a mask and is irradiated with ultraviolet light. The hydrogel cross-links only in the transparent areas of the mask to generate patterns similar to those of the mask. Khademhosseini and coworkers have intensively investigated the fabrication of cell-laden microgels for tissue engineering. They used this technique to create cell-laden microtissues and microfluidic devices.[100] Hydrogels can be patterned to create cellular microstructures for *in vitro* cell studies or 3D microtissues with biomimetic structures.

Because the complex architecture of most tissues is organized by assembly of repeating functional units over several scales, the cell-laden microgel units need to be assembled to larger structures at tissue level. Bottom-up assembly of cell-laden microgels has received increasing attention. These assembling techniques include random assembly,[101] manual manipulation,[102] multilayer photopatterning,[102,103] and microfluidic-directed assembly.[104,105]

Another approach is lamination of nanofiber layers with a hydrogel precursor solution followed molding to the final shape and cross-linking. In this approach, thin layers of nanofibers of PLGA, PLLA, PCL, or other polymers are produced by electrospinning. Then the fiber layers are laminated by compression molding using a hydrogel precursor solution containing bMSCs.[106] Then the laminated layer is wrapped around a cylindrical rod to form a microtubular osteon-mimetic structure and cross-linked by photopolymerization. The central canal in each microtube serves as a conduit for vascularization. A set of these microtubes can be adhesively bonded

to form a macroscale 3D cell-laden construct mimicking the microstructure of the cortical bone. This technique can potentially overcome the challenges associated with nonuniform cell seeding and vascularization and nutrient exchange within a bone-mimetic geometry.

However, these assembling techniques have their own drawbacks; they lack control over the final structure or lack scalability. Du et al. developed a more controllable assembling technique, which used hydrophobic effects in water–oil interfaces. Hydrophilic microgel building blocks microfabricated by photolithography were placed in hydrophobic medium and a secondary cross-linking reaction was performed.[105] However, this assembly technique exposed the microgels containing cells to the hydrophobic oil phase during the assembly procedure, which could influence cell viability.[105,107] Additionally, random or uncontrolled structures may form using this assembling approach. A recent work from Khademhosseini's group has modified the two-phase assembly technique using liquid–air interface of a hydrophobic solution to partially address the scaling-up issue by creating centimeter-scale cell-laden microgel assemblies.[92] However, this modified assembly was still performed in hydrophobic medium. To address this issue, directed assembly on hydrophilic templates was developed in the same group to fabricate 3D microgel constructs with a wide range of shapes and complexities such as tubes, spheres, and casques in 2D and 3D structures.[108,109] Other assembling techniques, such as physical templating and microfluidic-directed assembly, are also developed.[101,104,110]

Another novel technique in bottom-up TE approach is 3D cell, tissue, and organ printing. This technique is an attractive scaffold-free, rapid-prototyping based technology[111] with great potential for constructing delicate 3D tissue-like structures.[112,113] To engineer a bone tissue, osteogenic cell-laden hydrogels are deposited on a platform, yielding tissue constructs that consist of bone-forming cells and matrix at predefined locations within a porous 3D structure.[111]

Recently, Fedorovich et al. demonstrated the retention of spatially organized, functional osteogenic and endothelial progenitor cells, osteogenic matrix formation of hMSCs, and formation of erythrocyte-filled blood vessels in printed grafts after *in vivo* implantation.[114] SangJun Moon developed a bioprinter that used mechanical valves to print high-viscosity hydrogel precursor solutions containing cells within collagen, overcoming the problem of loss of cell viability and clogging in traditional inkjet printing systems.[115]

Cell bioprinting provides a potentially powerful technique in mimicking the native tissue microvasculature and microarchitecture, although the use of these implants still has limits in non–load-bearing applications. Temporary mechanical stability could be still required in combination with surgical instrumentation if applied in clinical environments.

Cell-sheeting techniques represent another bottom-up TE approach in which cells are grown on a thermo-responsive polymer substrate to secrete ECM and reach confluency. The confluent cell layer is detached by thermal regulation without enzymatic treatment, and single cell layers can be laminated into multiple single cell layers to form a thicker 3D matrix.[116] However, it is a challenge to construct thick tissues by this method because each layer is around 30 μm thick.[117,118]

Aside from the techniques discussed, cell aggregates are also a suitable building block for tissue-like constructs. The cell aggregates can be directly assembled into a tissue by using the adequate biological cues. Direct seeding of cell aggregates need to be in the presence of growth factors or other bioactive molecules to facilitate the dispersion of colonies into a larger cell construct.

6.3.2.3 Limitations and Challenges The bottom-up TE approaches hold great promise for creating functional repeating tissue units using hydrogels; they also provide a potential for assembling defined 3D microstructured modules for engineering tissue macroconstructs, which mimic the complexity of living tissues. However, random or uncontrolled structures still may form, so fabricating tissue constructs with biologically relevant length scales using the current setups is challenging.[119] Because of their high water content, hydrogels usually have poor mechanical stability. As a result, their use in constructing 3D tissues by bottom-up approaches is limited in load-bearing bone tissue. In addition, the control of the assembly process to fabricate 3D constructs with uniform shapes is still a challenge.

6.3.3 Novel Strategy (Integrating Approach)

6.3.3.1 Concept Top-down and bottom-up fabrication strategies have both advantages and disadvantages. The "challenges" of the bottom-up TE highlight the importance of scaffolds produced by traditional polymer processing techniques, such as porogen leaching and gas foaming. The lack of functionality of the top–down constructs underscores the importance of microenvironment for optimal cell growth. A combination of traditional top-down processes with more recent bottom-up microfabrication techniques may overcome this drawback and provide distinct advantages, bringing the field closer to the ultimate goal of complete control over microarchitecture and porosity in engineered tissues. The key question is how these two directions can be integrated. New strategies are still required to overcome the limitations of each of the current TE approaches.

6.3.3.2 Integrating Processes Mata et al. integrated top-down microfabrication with self-assembling peptide-amphiphile (PA) systems to offer a unique platform in which both physical and biomolecular elements were combined in a single material with cell behavior controlled by cell processing. In this integrated approach, bioactive scaffolds combine biologically instructive nanoscale fibers with topographical features to establish highly complex tissue structures.[120]

Ouyang et al. assembled a bMSC sheet on a knitted PLLA scaffold for engineering ligament analogs by a wrapping technique. Their results show that the approach of assembling bMSC sheets onto a knitted PLLA scaffold is promising for producing tissue-like and functional ligament analogs.[121]

Sargeant et al. developed a hybrid bone implant material consisting of porous Ti–6Al–4V foam and self-assembled PA nanofibers. Cells were encapsulated into the PA solution, and prewet Ti–6Al–4V foams with 52% porosity were placed in the PA and cell solution. PA solutions with cells were gelled with $CaCl_2$ to form nanofiber

matrices in Ti–6Al–4V foams. This hybrid bone graft, which integrated self-assembly of PA nanofibers within pores of metallic foams, has the potential to induce mineralization and direct a cellular response from the host tissue.[122]

Although the integration of bottom-up and top-down micro- and nanotechnologies brings new potentials to create tissue regeneration scaffolds with physical and biochemical hierarchical order from the micro- to macroscale, sophisticated technologies need to be developed. The major challenge of integration of bottom-up techniques with more traditional top-down approaches is to create more complex tissues than are currently achievable using either approach alone by optimizing the advantages of each technique.[123] Currently, there is no integrating technique that can be used to assemble complex hierarchical structures to meet the requirements of tissues and constructs, and research is now focused on targeting this problem.

6.4 FUTURE DIRECTION AND CONCLUDING REMARKS

Integration of a top-down TE approach with a bottom-up biological assembly concept is promising to engineer fully functional tissues and organs with micro- and nano biomimetic hierarchical complexity. Each approach has its own strengths and weaknesses and is suitable for different TE applications. The continuous development of top-down TE techniques will improve the scaffold's microstructure, presentation of cell signaling factors, and the interaction between multiple types of cells. The improvements in bottom-up approaches will generate novel self-assembling building blocks and complex larger scale tissue structures. With continued research in these advanced techniques, bone tissue engineering will advance toward clinical restoration of tissue function. Advances in top-down and bottom-up approaches will improve scaffold mechanical properties, cell–cell and cell–matrix interactions, and cell shape and morphology, leading to the formation of a vascular mineralized matrix in the damaged tissue and greater integration of the construct with the host vasculature.

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