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Biomimetic Scaffolds for Tissue Engineering

Taek Gyoung Kim, Heungsoo Shin, and Dong Woo Lim**

This article is dedicated to Professor Tae Gwan Park, whose wisdom and passion for pioneering biomaterials will be remembered forever in our hearts to make the world a better place

Biomimetic scaffolds mimic important features of the extracellular matrix (ECM) architecture and can be finely controlled at the nano- or microscale for tissue engineering. Rational design of biomimetic scaffolds is based on consideration of the ECM as a natural scaffold; the ECM provides cells with a variety of physical, chemical, and biological cues that affect cell growth and function. There are a number of approaches available to create 3D biomimetic scaffolds with control over their physical and mechanical properties, cell adhesion, and the temporal and spatial release of growth factors. Here, an overview of some biological features of the natural ECM is presented and a variety of original engineering methods that are currently used to produce synthetic polymer-based scaffolds in pre-fabricated form before implantation, to modify their surfaces with biochemical ligands, to incorporate growth factors, and to control their nano- and microscale geometry to create biomimetic scaffolds are discussed. Finally, in contrast to pre-fabricated scaffolds composed of synthetic polymers, injectable biomimetic scaffolds based on either genetically engineered- or chemically synthesized-peptides of which sequences are derived from the natural ECM are discussed. The presence of defined peptide sequences can trigger in situ hydrogelation via molecular self-assembly and chemical crosslinking. A basic understanding of the entire spectrum of biomimetic scaffolds provides insight into how they can potentially be used in diverse tissue engineering, regenerative medicine, and drug delivery applications.

1. Introduction

Since the early 1990s when the concept of tissue engineering was first developed, a variety of tissue engineering applications have been developed by introducing cells, genes, proteins, or other biological molecules into a 3D porous matrix known as a scaffold.[1] While enormous progress has been made in designing and engineering scaffolds, biomimetic scaffolds, which have extracellular matrix (ECM)-mimicking architectures have recently emerged as a promising class of materials for tissue repair. Rational design of biomimetic scaffolds is based on the natural scaffold of the ECM, which provides cells with a variety of physical, chemical, and biological cues that determine cell growth and function.[2,3] Therefore, to create an optimized cellular microenvironment conducive to the growth of 3D structured tissue, biomimetic scaffolds with controllable physical and mechanical properties, cell adhesion properties, and growth factor release kinetics are required.

The breakthrough in engineering biomimetic scaffolds has been driven by the following scientific developments: i) prefabrication of 3D scaffolds before implantation to target tissue with controlled ECM-like structures due to the use of biomaterials, incorporation of porosity, and nano- and micrometer-scaled topologies; ii) surface modification and biofunctionalization of the scaffolds to mimic the biologically relevant environment; and iii) formation of in situ gelled hydrogel scaffolds composed of rationally designed, biologically inspired materials via physical- or chemical crosslinking. Pre-fabricated scaffolds or in situ gelled hydrogel scaffolds are introduced into tissue defects, either alone or with target cells with regenerative potential for tissue repair. Generally, by incorporating bioactive molecules into scaffolds and allowing easy removal of unreacted residues and ex vivo culture of cells under defined conditions, pre-fabrication methods can be used to control the post-modification of scaffolds although they may not completely recapitulate the 3D environment of the ECM. In contrast, as compared to pre-fabricated scaffolds, in situ gelation by physically or chemically crosslinked networks holds many advantages: it can be applied as an injectable system to the target lesion in a relatively minimally invasive manner and target cells can be in situ encapsulated within scaffolds and eventually surrounded within the natural ECM-like microenvironment. Many hydrogel scaffolds have been developed from

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synthetic and natural polymeric materials as truly artificial ECM-like scaffolds. Recently, genetically engineered- or chemically synthesized peptides of which sequences are derived from native proteins have attracted great interest for fabrication of injectable biomimetic scaffolds since they are easily 3D assembled via molecular self-assembly and chemical crosslinking under physiological conditions. In addition, extra biofunctionalization steps are unnecessary due to the ability to precisely incorporate functional peptide sequences that are rationally designed at the molecular level.

This article is divided into three general themes in preparation of biomimetic scaffolds. Firstly, we present an overview of some biological features of the natural ECM. General approaches in fabrication of biomimetic scaffolds rely on identification of specific biomolecular components in ECM and understanding of their interactions with cells and tissues. We then describe a variety of original engineering methods that have been used to produce scaffolds as a pre-fabricated form with diverse three-dimensional morphology, modify their surfaces with biochemical ligands, present growth factors, and control their nano- and microscaled geometry for creating biomimetic scaffolds. Finally, we discuss the rational design, synthesis, and in situ gelation mechanism of biologically inspired peptide-based materials that can be used as injectable biomimetic scaffolds.

2. The ECM as a Natural Scaffold

2.1. Composition and Structure of the ECM

Cells within 3D tissues are surrounded by ECM components and bioactive molecules such as proteins, glycosaminoglycans (GAGs), and glycoproteins (GPs), as shown in **Figure 1**.[4] Major proteins in the ECM include elastin, collagen, fibronectin, and laminin, and their function and composition vary for each tissue type. Elastin is found in large quantities in tissues requiring elastic properties such as artery and bladder tissues.[5] Collagen is abundant in bone and skin tissue where it is assembled into fibrillar structures that convey tensile strength. Collagen has many bioactive domains, and Gly-Phe-hydroxyPro-Gly-Glu-Arg in the form of triple helical structures is one of the more common peptide sequences and is actively involved in interactions with cell membrane receptors.[6] An additional important bioactive protein is fibronectin, which exists as two linear chains linked to each other via disulfide bridges and contains collagen-binding, heparin-binding, and cell-binding domains.[7] Laminin, which forms a crosslinked web, is the main component of basement membranes.[5] However, the majorities of GAG components are not directly related to cell fate processes, but provide a structural network. Highly sulfated and negatively charged functional groups in GAG molecules maintain a high water uptake ratio; therefore, the main function of GAGs is to determine tissue resilience, although they do have other roles. Several growth factors such as fibroblast growth factor, vascular endothelial growth factor, and platelet-derived growth factor display multiple heparin-binding domains that are capable of binding to heparin sulfate or heparin with high affinity and specificity.[8] It should be noted that GAG molecules can control the storage and liberation of growth factors, and GAG-growth factor interactions protect growth factors

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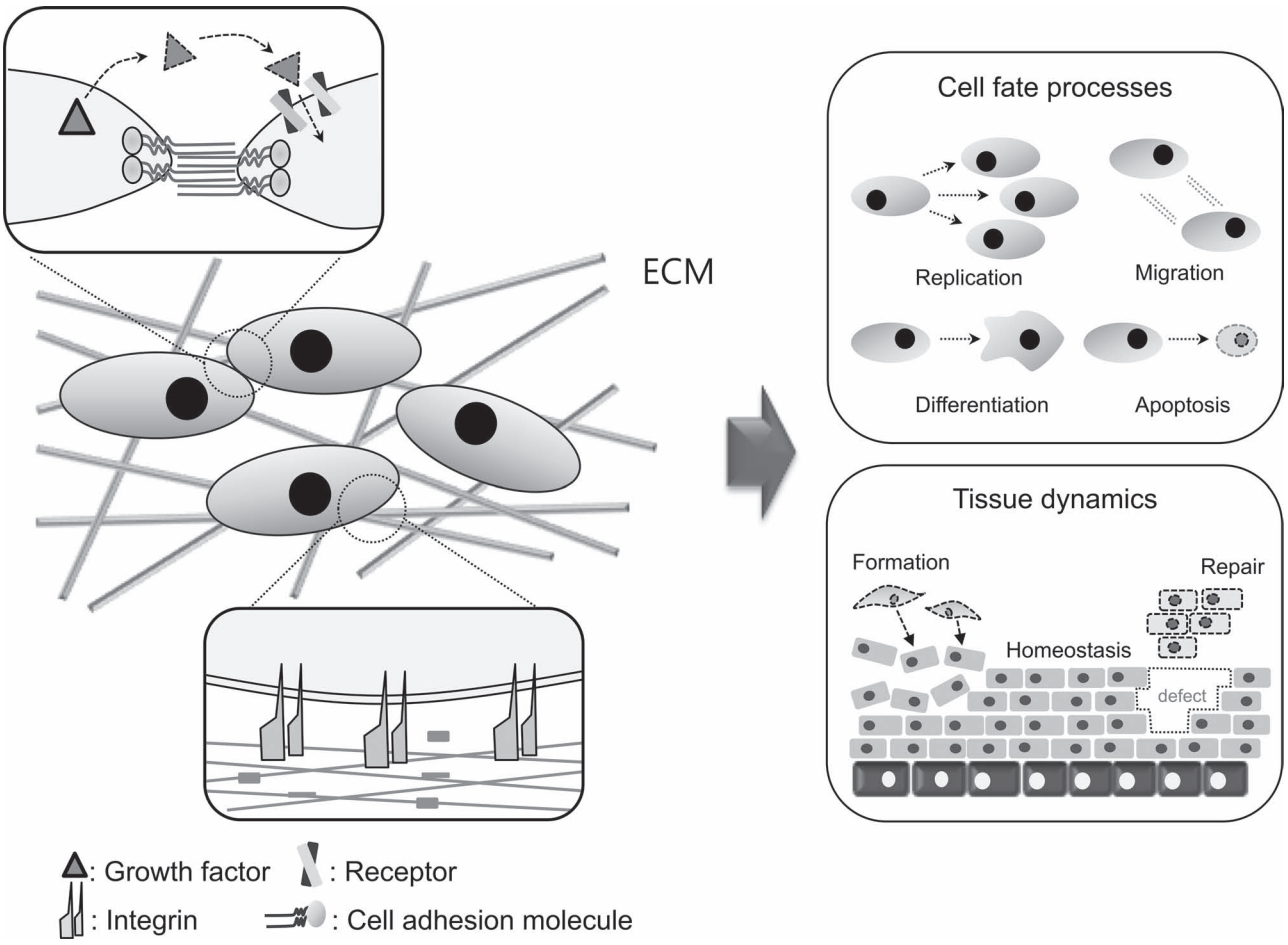

Figure 1. Structure, composition, and functional roles of the ECM milieu. Cells reside within a 3D fibrous assembly comprised of numerous biomolecules including proteins, glycosaminoglycans, and proteoglycans. The ECM provides structural support for cell adhesion, which triggers downstream cell signaling pathways. In addition, the ECM serves as a reservoir of soluble growth factors and regulates the reversible presentation of these growth factors and prevents their proteolytic degradation. Interactions of cells and ECM components with diverse chemical compositions and micro/nanoscaled topographies control cell fate processes including adhesion, migration, proliferation, differentiation, and apoptosis. Furthermore, macroscopic tissue dynamics including tissue development, homeostasis, and repair may also be modulated by these interactions.

from proteolytic degradation. Therefore, scaffolds should provide a microenvironment for cells similar to that provided by the native ECM, and emulation of the bioactivities of proteins as well as the ability of the ECM to store and sequester growth factors should be considered when developing biomimetic scaffolds.

The primary role of the ECM is to provide structural support to cells, and the composition and local distribution of ECM components dictate the 3D architecture of the ECM. In principle, the ECM consists of complex fibrillar networks composed of fibrous proteins and GAGs that are chemically crosslinked or physically bound due to homotypic or heterotypic interactions between molecules, or both.[9] The concentration of assembled proteins and GAGs determines the geometry, topography, porosity, density, and mechanical stiffness of the ECM network, which further regulate many cell fate processes. For example, an increase in the concentration of collagen could retard cell migration and trigger hypoxia-related signaling processes because it leads to a highly crosslinked ECM network and thereby limits the supply of gases and nutrients.[10] Recent studies have highlighted the importance of matrix density as an instructive signal for stem cell differentiation; mesenchymal stem cells preferentially differentiated into neuronal, myogenic, and osteogenic tissues when cultured on hydrogels with the lowest (0.1 kPa), intermediate (11 kPa), or highest (34 kPa) stiffness, respectively.[11] In addition, micro/nanoscaled topography created by organized fibrillar bundles appears to have a profound impact on cell shape, organization of the cytoskeletal structure, and intercellular signaling.[12] It is also worth noting that cells release many proteases in response to physiological signals, and these proteases dynamically remodel ECM structures by changing the local modulus and chemical composition of the ECM.[2] Therefore, defining the 3D architecture of the ECM and the interactions amongst ECM components in each tissue type is the first step toward designing biomimetic scaffolds.

2.2. Cell Adhesion to the ECM

The majority of cells are anchorage-dependent and therefore the coordination of cell fate processes including proliferation,

migration, apoptosis, and differentiation is highly influenced by cell adhesion to cell-binding epitopes in the ECM. The primary cell adhesion receptors are integrins, which are composed of two transmembrane α and β subunits. Currently, 18 α, 8 β subunits, and 24 distinct heterodimeric combinations have been identified, and each of these integrins has distinct specificity toward cell-binding ligands.[13] For example, integrins $\alpha_5\beta_1$ and $\alpha_5\beta_3$ are responsible for binding[14] to fibronectin while integrin $\alpha_2\beta_1$ binds to collagen.[15] Upon physical binding of integrins to ligands, the integrins cluster and structural proteins within the cytoplasm such as vinculin, talin, and α-actinin are localized to form a premature adhesive junction between intercellular and extracellular molecules. At the same time, signaling proteins in the cytoplasm associated with the adhesive complex trigger downstream signaling events. The mature state of the cell adhesion complex that comprises ECM ligands, integrins, and cytoskeletal proteins is known as a focal adhesion (FA), which is a critical structure required by cells to sense external chemical, biological, or mechanical stimuli.[16] Robust formation of a FA is generally indicative of activation of many cell survival signals, and therefore many biomimetic strategies have targeted the control of FA formation and related signaling processes. Cell adhesion to ECM proteins does not require the presence of entire macromolecular chains of proteins, but specific peptides sequences along the protein backbone can be recognized and bound by integrins with the same affinity and specificity. For example, short peptides of Arg-Gly-Asp (RGD),[17] Pro-His-Ser-Arg-Asn (PHSRN),[18] and Arg-Glu-Asp-Val (REDV)[17] derived from fibronectin, Gly-Phe-Hyp(hydroxyproline)-Gly-Glu-Arg (GFOGER)[15] derived from collagen, and Ile-Lys-Val-Ala-Val (IKVAV) and Tyr-Ile-Gly-Ser-Arg (YIGSR) derived from laminin[4] can be recognized by cell membrane receptors. Therefore, both finely controlled composition and 3D location of various ligands within scaffolds are critical factors for biomimetic scaffold fabrication.

3. Biomimetic Scaffold Fabrication

In a living system, cells collect a variety of instructions through communication with the adjacent cells within tissues and, more importantly, cellular behaviors are governed by complex biochemical and biophysical information presented by the ECM, as exemplified by cell fate determination and contact guidance phenomenon.[19–21] Cells reside in a 3D cushioned network and are protected against external mechanical stress.[22–24] Moreover, the ECM provides a proper niche for cell adhesion, migration, proliferation, and differentiation due to molecular interactions between specific cell membrane receptors and signaling cues from surrounding ECM materials.[25] Therefore, for successful tissue regeneration at a target site, the architecture of scaffolds should emulate the natural design of the ECM and instruct cellular behavior while adequately housing the cells.[26,27] Tissue engineering (TE) scaffolds have been prepared using a variety of materials and various processing conditions; employment of appropriate fabrication methods is critical for successful regeneration of target tissue. Generally, TE scaffolds are produced as 1) pre-fabricated scaffolds or 2) in situ gelled hydrogel scaffolds. In this section, we discuss several representative processes used to produce the various types of TE scaffolds as the pre-fabricated form, modify their surfaces with biochemical ligands, present growth factors, and control their nano- and microscaled geometry.

3.1. Preparation of Pre-Fabricated Scaffolds

Until recently, conventional approaches to produce pre-fabricated scaffolds have focused mainly on introduction of open pores and inter-connected channels within biodegradable scaffolds to increase the viability of seeded or injected cells.[28,29] Various processing techniques have been developed to fabricate biodegradable 3D foams for scaffolds. In particular, salt leaching and gas foaming methods have been widely used because these methods are straightforward, cost-effective, and easy to scale-up.[30–32] Numerous scaffolds prepared from these techniques have resulted in successful clinical outcomes and are eventually expected to enter the commercial market.[33] As an alternative, electrospinning allows nanoscaled fibrous design of scaffolds that mimic functional collagen structures.[34–37] Electrospun nanofibers are characterized by complex fibrous and interconnective porous structures, which make it possible to fabricate highly structured scaffolds for inducing cellular growth. Microfabrication techniques are based on rapid prototyping (RP) methods including stereolithography, selective laser sintering, shape deposition manufacturing, fused deposition modeling, and 3D bioplotting, and these are considered as effective routes to fabricate custom-made scaffolds with a given anatomical contour for hard tissue regeneration.[38–41] Recently, modular assembly methods have been used to produce biomimicking hybrid tissues with a synthetic architecture loaded with inductive biological signals and specified cells.[42,43]

3.1.1. Porous Scaffolds by Conventional Techniques

Biocompatible and biodegradable polymeric foams have been harnessed as temporal structural supports to regenerate various tissues such as bone, cartilage, nerve, ligament, skin, and liver. An open porous geometry with interconnected channels is a prerequisite for high density cell growth within the scaffold as well as the mass transport of nutrients, oxygen, and metabolic waste; a high cell density and efficient mass transport contribute to cell viability, proliferation, and ultimate rehabilitation into functional tissues.[41,44] Bioerodible aliphatic polyesters including poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymer, poly(lactic-*co*-glycolic acid) (PLGA) have been widely used to fabricate biodegradable scaffolds because of their controllable hydrolysis into nontoxic components in vivo.[45,46] A wide range of biodegradable scaffolds with diverse morphologies have been fabricated by conventional methods such as porogen leaching, gas foaming, emulsion/freeze drying, and expansion in supercritical fluid.[47] In particular, a porogen leaching method was primarily employed to produce a highly porous scaffold with large interconnectivity.[48–51] In this method, particulate materials at a micrometric level including inorganic salts, carbohydrates, and paraffin spheres are usually embedded into a polymer/solvent paste, followed by elimination through washing after solvent evaporation.

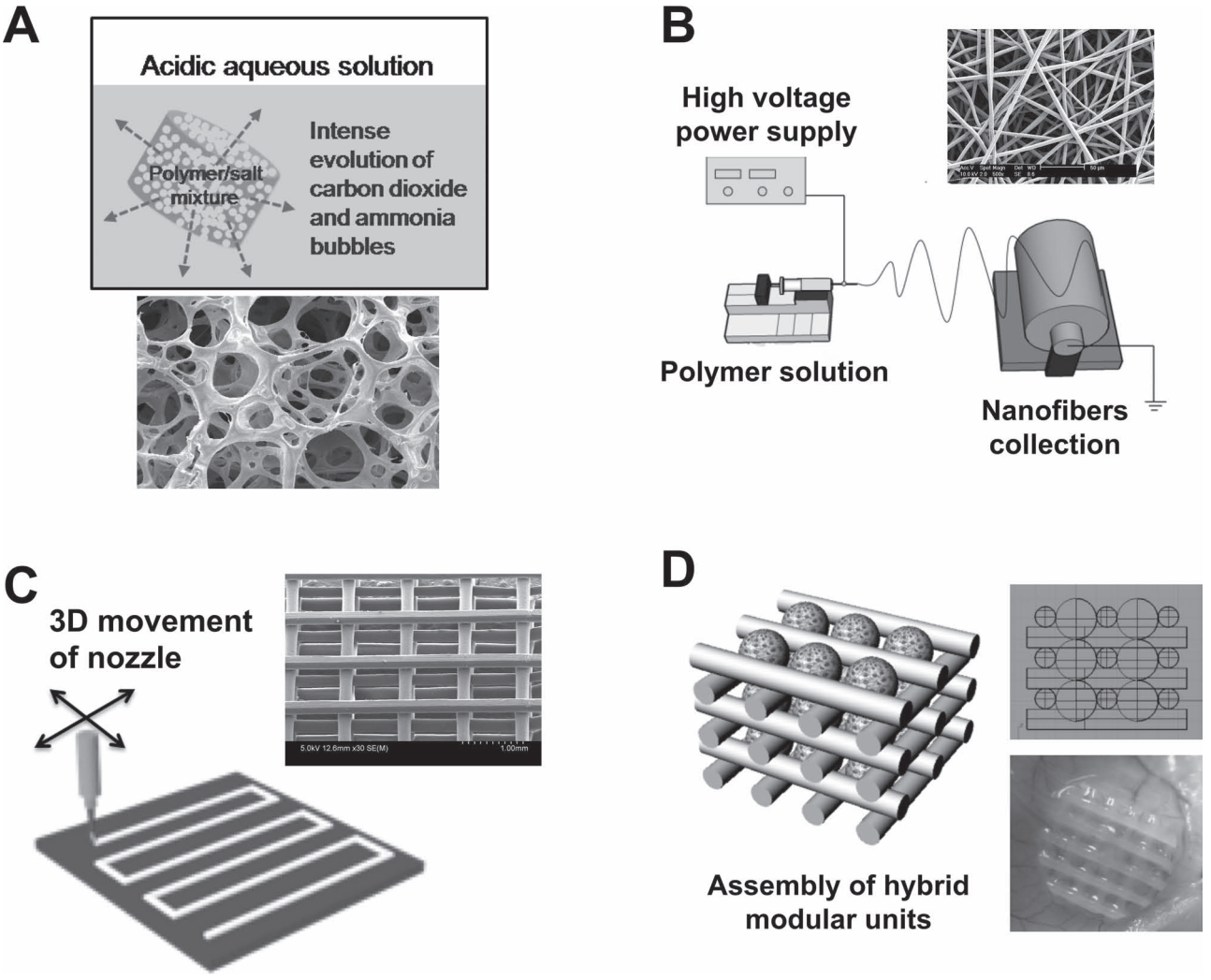

Figure 2. Different technological approaches used to produce polymeric scaffolds for tissue engineering. A) Highly porous scaffolds are fabricated by gas foaming and salt leaching. B) Electrospinning is an ultrathin fiber-forming technique that uses an electrostatic force. C) Rapid prototyping allows production of 3D porous scaffolds with defined and regular pore structures. D) An adipose tissue construct is prepared by modular assembly using multicellular spheroids with the aid of polymeric templates.

Park and co-workers previously produced macroporous PLGA scaffolds by a gas foaming/salt leaching method (**Figure 2**A).[29,52] Effervescent salt porogens, such as ammonium bicarbonate or sodium bicarbonate, were uniformly mixed with precipitated, semisolidified PLGA clumps in a nonsolvent. Once the polymer/salt mixture was submerged in an acidic aqueous solution, intense evolution of carbon dioxide and ammonia bubbles originating from effervescent salts embedded in the polymer paste created large open pores throughout the scaffold body. These porous scaffolds allow a high cell population density, and therefore show promise for reconstruction of liver and cartilage tissues.[53–55] Highly open porous PLGA microspheres have been prepared by employing ammonium bicarbonate as a gas foaming agent using the sophisticated approach (Figure 2A).[56] Ammonium bicarbonate solution was incorporated into a primary water-in-oil emulsion, and subsequently generated bubbles, leading to emulsion stabilization. Through solvent evaporation at the second emulsion stage, an internal pore structure filled with water and gas was solidified to produce a spherical, porous scaffold. NIH3T3 cells (mouse embryonic fibroblast cell line) proliferated on the porous microspheres, and high density cell growth was achieved by infiltration of the cells into the internal pore spaces. In another study, the degree of porosity and the overall pore size as well as the surface properties of the microspheres were finely tuned.[57] The mean pore size of the microspheres was adjusted from 7.9 μm to 29.4 μm by varying PLGA concentration in oil phase during double emulsion process. Furthermore, the hydrophilic amino groups were introduced on the microsphere surface to facilitate the cellular adhesion and proliferation. When chondrocytes were cultured on the superporous microspheres, large amounts of deoxyribonucleic acids, glycosaminoglycans, and collagen were produced, implying a high rate of cell growth and expression of a cartilage-specific phenotype. These porous spherical scaffolds could potentially be used to deliver therapeutic cells to damaged sites by injection.

3.1.2. Fibrous Matrices by Electrospinning

Electrospinning is a versatile tool for producing ultrafine fibers with a wide range of diameters from a few micrometers to several nanometers. The externally applied Coulomb forces are comparable to the mechanical forces applied in traditional spinning (Figure 2B). The processing flexibility of this technique ensures fiber production from a broad range of precursor materials that include synthetic polymers, natural polymers, semiconductors, ceramics, or their combinations.[58] The final product formed by electrospinning is often in the form of a 2D nonwoven mesh characterized by randomly oriented nano- or micrometer sized fibers. Electrospun fibrous meshes have fascinating characteristics such as an extensive surface-to-volume ratio and a highly interconnective porous architecture, and can be assembled into aligned fibers, making these meshes ideal for application in nanoscaled devices.[59]

Compared to the conventional methods producing porous materials, during past several years, the electrospinning technique has received more attention because of simple and powerful means to produce nanometer-sized elements.[60] With regard to biomedical applications such as TE scaffolds, drug delivery carriers, and wound care devices, these fibrous materials allow delicate modulation of cellular behaviors and fine control of drug release. Because of their structural and morphological similarity to the native ECM, electrospun nanofibers have been exploited to attempt to create an ideal TE scaffold. A variety of synthetic polymers such as biodegradable aliphatic polyesters and native biopolymers, for example, collagen, silk fibroin, chitosan, alginate, and hyaluronic acid, have been electrospun as biomimetic and temporal substrates to modulate various cellular activities.[61–63] For instance, the natural collagen fiber analogue generated by electrospinning served as an excellent substrate for endothelial cell growth and osteoblast differentiation.[64,65] Electrospun hyaluronic acid also revealed the ability to facilitate chondrocyte growth whilst the cell retaining typical cartilaginous phenotypes, showing promise in the application of cartilage regeneration.[66]

However, transformation of natural polymers into nanofibers by electrospinning has been plagued by high variability and low reproducibility during processing. In contrast, synthetic polymers without positive biochemical signals exhibit easy processability and controllable characteristics. In particular, nanofibers electrospun from synthetic polymers have been modified with a wide variety of bioactive molecules such as fibronectin, laminin, and various growth factors based on the fact that the tissue regeneration process is governed by biochemical cues on the surface where cells directly communicate. Consequently, surface modification regimes have succeeded in guiding cells into specific phenotypes and organizations.[67,68]

However, despite numerous benefits, it has been extremely difficult to design macroscopically porous 3D architectures by using nanofibers, which are characterized by entangled fibers and densely packed membranous structure. Therefore, Zhang et al. used a novel 3D collecting template based on manipulation of electric fields and forces to control the ultimate 3D architecture; this approach generated electrospun tubes with diverse tubular configurations.[69] In another study, nanofiber aggregates were uniformly dispersed in aqueous solution by aminolysis degradation of long electrospun fibers for reassembly of the fibers with controllable orientation and architecture.[70] Cylindrical and biodegradable nanomaterials with various aspect ratios were prepared from transverse fragmentation of semicrystalline PLA nanofibers. This novel top-down approach allowed the fabrication of ECM-mimicking nanofilaments, which could potentially be assembled into highly ordered structures.

3.1.3. Rapid Prototyping-Based Microfabrication

Rapid prototyping (RP) techniques are defined as automated deposition of each tomographic layer sequence based on programmed 3D images into the ultimate desired architecture through additive layer-by-layer fashion (Figure 2C).[71,72] The RP technique is one of the most precise and reproducible avenues for controlling the internal pore size, porosity, pore interconnectivity, mechanical performance, and overall dimensions of TE scaffolds.[41] These features allow the scaffolds to be biomimetic to native tissues or organs with regard to shape and mechanocompatibility.[73–75] However, microfabricated products often lack the bioactivity to induce tissue regeneration. Because product fabrication is mostly implemented at the micrometer scale, integration of nanoscale features into the microstructure is necessary to finely tune cellular responses.[41]

Park and co-workers reported production of a dual-scale scaffold by combining the processes of RP and electrospinning.[76] In this process, the microfibrous layer of the scaffolds was first built via the RP process and then polymeric nanofibers were directly deposited onto the microfibrous layer by electrospinning. The subsequent microfibrous layers integrated with electrospun nanofibers were repeatedly laminated onto the previously integrated layers so that a 3D hybrid structure could be fabricated. The hybrid construct consisted of a microfibrous woodpile structure and electrospun nanofibers from different biocompatible materials, namely poly(ε-caprolactone) (PCL) and collagen. The dually designed scaffold exhibited improved biological function in terms of chondrocyte adhesion and proliferation. In another study, a layer-by-layer (LbL) polyelectrolyte assembly system was applied to a RP-based microstructured scaffold for application in bone tissue engineering.[77] Hydroxyapatite and collagen are the major extracellular components of natural bone tissue and have osteogenic effects on human mesenchymal stem cells (hMSCs). In this study, hydroxyapatite nanoparticles were first modified with catechol-functionalized hyaluronic acid. The modified hydroxyapatite nanoparticles with a negative charge and type I collagen with a positive charge were used as building blocks for LbL assembly, which led to the formation of a nanocomposite multilayer on the surface of the RP scaffold. While adjusting the amounts of hydroxyapatite and collagen on the surface, the degree of hMSC differentiation was monitored by measuring alkaline phosphatase (ALP) activity and the expression level of osteoblast-related genes, including bone sialoprotein-II (BSP), bone morphogenetic protein-2 (BMP2), osteopontin (OP), and osteocalcin (OC). ALP activity and the expression of the relevant genes increased in a pattern consistent with the levels of the two surface components. The success of a biomimetic design strategy that combines biomimetic structures with surface chemistry opens up new frontiers

in the fabrication of multifunctional scaffolds and also offers versatility in that the incorporation of other bioactive agents onto the scaffold such as bone growth factors, could further promote bone tissue regeneration.

3.1.4. Modular Hierarchical Assemblies

To date, producing large engineered tissues using pre-fabricated scaffolds has been limited by the non-homogeneous distribution of cells within the scaffold and a dearth of vascularization, leading to necrosis in the core of the scaffold. To address this issue, the strategy of modular tissue engineering, which aims to assemble biological modules typically consisting of cell masses or cell/polymer constructs, was introduced.[43] A modular scaffold is designed to retain flexibility and versatility for tissue reconstruction so that large complex tissues can be built by the connection of simple modular units in numerous different ways, and various modules with different functionalities can be incorporated into the final construct. Multicellular spheroids can be used as modular building units for tissue engineering and were previously self-assembled into a toroidal 3D structure using tissue liquidity mediated by cell–cell and cell–matrix interactions.[78–80] In addition, organ printing is an automated system that uses bio-ink to assemble modules into 3D functional living structures; for example, multicellular spheroids have been assembled with the aid of ECM-mimicking hydrogels.[81,82] However, the creation of a stable and durable bioconstruct is a major challenge for successful implantation and functional retention of the engineered tissue.

As reported previously, the nanofilaments mentioned above were modified with the RGD peptide moiety and self-assembled with hMSCs to form composite multicellular spheroids.[83] These composite spheroids showed enhanced hMSC viability and a high level of adipogenic differentiation compared to blank spheroids composed only of cells. These optimized composite spheroids were used as modular building units for adipose tissue engineering. Microfibrous templates produced by the RP technique were used to mechanically stabilize the assembled tissue. The microfibrous templates were also modified with an angiogenic growth factor, namely basic fibroblast growth factor (bFGF), to induce neovascularization. These bioactive templates were utilized to assist the 3D assembly of composite spheroids (Figure 2D), yielding a biomimetic hierarchical assembly with solid multiscaled features. Implantation of the resulting engineered tissue construct in the dorsal subcutaneous pockets of nude mice resulted in the successful formation of functional and vascularized adipose tissue, implying that by retaining the prescribed tissue contours and adipose tissue function, the main hurdles in regeneration of soft tissue were overcome. This assembly concept and modular design can be extended to reconstruct a variety of multifunctional tissues and organs.

3.2. Functionalization of Pre-Fabricated Scaffolds

3.2.1 Surface Modification of Biomimetic Scaffolds

Although many biodegradable polymers such as PLA, PGA, PLGA, and PCL have been used to create controllable structures for directing the repair and regeneration of damaged tissues, active control of cell adhesion and down-stream cellular events is still challenging due to the absence of biofunctional ligands that can instruct cells.[17,84] Therefore, many efforts have been made to incorporate bioactive ECM molecules onto the surfaces of scaffolds using various modification modalities including non-covalent and covalent binding. ECM proteins can be non-covalently adsorbed onto the surfaces of scaffolds by electrostatic interaction and van der Waals forces. Fibronectin and laminin are used to coat PLLA and PCL scaffolds and have been shown to improve the cell adhesion of many cell types.[85,86] In addition, stem cells appear to preferentially differentiate into osteoblasts depending on the type of ECM adsorbed to the scaffolds.[87] A recent ECM microarray study investigated the effects of combination of ECM molecules such as collagen-I, III, IV, laminin, and fibronectin on selective differentiation of embryonic stem cells, and the authors of that study highlighted the importance of ECM proteins as immobilized biological cues on scaffolds.[88] However, physical adsorption is a relatively weak force and may therefore not be appropriate for tissue engineering applications for which prolonged signaling is required.

As an alternative, covalent immobilization of bioactive molecules has been achieved by surface etching[83] and plasma/gamma-ray treatment,[89] which involves the cleavage of a degradable polymer backbone to generate carboxyl (–COOH) groups or radicals, respectively. These functional groups can undergo further reactions with ECM proteins (fibronectin, collagen, and gelatin) and related bioactive peptides (e.g., RGD, YIGSR, IKVAV, DGEA) on the surfaces of various scaffolds including PLLA, PVDF, PCL, and PLCL.[90,91] The covalently conjugated molecules have been shown to be stable under physiological conditions and maintain biological activity after prolonged implantation in the localized microenvironment. Initially, these bioactive ligands improved cell adhesion to the surface and were engaged in cell-specific signaling. Fibronectin conjugated to PLLA, PCL, and silicone rubber scaffolds significantly enhanced the survival of osteoblasts,[92] chondrocytes,[93] and myoblasts,[94] while collagen-modified PLLA scaffolds facilitated the proliferation of chondrocytes for 20 days. RGD peptide-immobilized scaffolds facilitated osteogenic differentiation of osteoblasts and the myogenic differentiation of muscle precursor cells. Similarly, IKVAV[95] and YIGSR[96] have been reported to regulate neural and endothelial cell differentiation, respectively. The density and distribution of bioactive molecules presented from the surface should be optimized for appropriate control of cell behavior. A high density of bioactive molecules on the surface is related to greater cell spreading, focal contact formation, and proliferation to a certain extent, whereas cell migration is proportional to protein density on the surface in a bi-phasic manner.[97] Therefore, a minimal amount of signaling peptide can be generally used for surface immobilization; a previous study reported that as low as 1 fmol cm^{-2} and 10 fmol cm^{-2} of RGD peptide was sufficient for cell spreading and focal contact formation, respectively. In addition to peptide density, the distribution of the peptide on the surface regulates integrin clustering. Given that the size of integrins is approximately 10 nm and their clustered complex structure is less than 100 nm, the distribution of peptides for cell-adhesive mimetics should be considered on a nanometer scale.[98] Recently, a

versatile technique to immobilize bioactive molecules based on mussel adhesive proteins was reported. To demonstrate the utility of this technique, dopamine was polymerized onto metals, polymers, and ceramics, and then secondary ligands, for example, ECM bioactive molecules, were covalently immobilized to the polydopamine layer.[99–101] Similarly, another study utilized hybrid mussel adhesive proteins fused with short ECM bioactive domains using recombinant DNA technology.[102] These approaches are effective in that surface immobilization can be carried out irrespective of the chemical composition of the materials.

3.2.2. Biomimetic Scaffolds Presenting Growth Factors for Controlled Release

Growth factors are key biochemical cues that trigger intracellular signal cascades required for tissue regeneration and are therefore generally incorporated into scaffolds where they can deliver positive biochemical signals ranging from promotion of mitogenic activity to induction of neovascularization.[103–105] The growth factors used for construction of engineered tissues are strongly dependent on the target tissue types to be regenerated. Epidermal growth factor (EGF), fibroblast growth factors (FGFs), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF) are frequently used to accelerate wound healing because of their mitogenic induction of epithelial cells and fibroblasts as well as up-regulation of matrix formation.[106–108] For example, chemical immobilization of recombinant human EGF (rhEGF) on an electrospun scaffold was successfully employed for wound healing.[109] The rhEGF-modified nanofibrous scaffold preserved the growth factor activity even under harsh conditions such as enzymatic degradation, consequently inducing keratinocyte differentiation. Bone morphogenic protein-2 (BMP-2) and TGF-β1 are often required to induce and maintain tissue-specific properties for regeneration of hard tissues such as bone and cartilage.[110–112]

The TE scaffolds can be implanted with or without embedded cells. In one study, cell-loaded scaffolds showed adverse effects leading to poor healing processes as a result of immune responses triggered by the ECM materials secreted from the pre-seeded cells.[113] The use of cell-free scaffolds may therefore be more desirable in certain cases. If using a scaffold-only approach, the scaffold has to be highly modified with bioactive molecules to induce the infiltration of peripheral progenitor cells or multipotent stem cells. The regenerative conductivity or inductivity directed by the scaffold is mainly due to the composition of the material, which can deliver biochemical cues such as growth factors.[114] To repair bone lesions, growth factor-releasing scaffolds have been employed to ameliorate osteogenesis and accelerate vascularization, both of which can be regulated by the dose and release kinetics of the growth factors.[115] Specially, collagen sponges incorporating recombinant human BMP-2 (rhBMP-2), marketed as the INFUSE Bone Graft, are clinically used for treating degenerative disc diseases. In a human clinical trial, the rhBMP-2/collagen sponges exhibited more reliable osteoinduction than autogenous bone grafts.[116]

The shortage of autologous cell sources for tissue reconstruction has led to attempts to use pluripotent or multipotent stem cells.[117,118] Specifically, MSCs are most often used as an alternative cell source for reconstructing connective tissues such as cartilage, bone, adipose, and tendon. Studies that have used progenitor cells or stem cells for tissue engineering have reported that scaffolds laden with growth factors result in a high degree of cell differentiation. Typically, prolonged release of growth factors and a short diffusion distance between a bound growth factor and a target cell are required to maximize the activity of the growth factors because of their short biological half-lives in the body.[119–121] For example, gelatin microspheres loaded with TGF-β3 were used to induce the assembly of hMSCs into cellular aggregates.[122] Diffusion of TGF-β3 released from microspheres into the hMSCs of cellular aggregates stimulated chondrogenesis, as assessed by the amount of DNA, glycosaminoglycan (GAG), and collagen type II produced. In addition, two types of electrospun nanofibers with different bFGF release profiles and surface hydrophilicity were compared by measuring the differentiation level of hMSCs.[121] Stem cells grown on electrospun fibers with fast release of bFGF and a more hydrophilic surface showed increased collagen production and up-regulated gene expression, consistent with fibroblastic differentiation, implying that the release kinetics of the growth factor as well as the surface properties of the scaffold could alter stem cell fate. Furthermore, proper infiltration of blood vessels allows long-term survival of the implanted tissue construct by promoting transport of indispensable nutrients, oxygen, and cell-signaling molecules. Angiogenic growth factors including acidic or basic FGF (aFGF or bFGF), vascular endothelial growth factor (VEGF), angiopoietins, and PDGF can be incorporated into scaffolds to facilitate host tissue ingrowth and induce angiogenesis.[123,124] PLGA foams fabricated by using solvent casting with salt leaching or supercritical carbon dioxide have been employed to directly encapsulate bFGF.[125] Protein release was fairly constant, but an initial burst and relatively low loading efficiency were unavoidable. Therefore, heparin and heparin sulfate have been exploited to immobilize bFGF on the surfaces of various scaffolds on the basis of the strong binding affinity between heparin and bFGF (**Figure 3**A).[83,126,127] The heparin-mediated delivery of bFGF has distinct advantages in terms of protection of early degradation of growth factors and facilitation of cell recognition, ultimately resulting in better angiogenesis (Figure 3B). Chemically heparinized foams subsequently loaded with bFGF or VEGF have proved to be an efficient delivery platform for growth factors, with stable and sustained release of growth factors over an extended period and consequently superior angiogenic activity. Heparinized electrospun fibers also showed a high degree of bFGF surface immobilization and more prolonged release of the growth factor than that of bare electrospun fibers.[83] Similarly, laminin and bFGF were simultaneously adsorbed on heparinized nanofibers.[128] The combination of immobilized biochemical cues, i.e., laminin and bFGF, as well as nanofiber alignment, synergistically induced oriented neurite outgrowth, and cell migration. In addition to localized introduction of a single growth factor, delivery of multiple growth factors in simultaneous or sequential release mode can enhance treatment efficacy. A polymeric system for dual growth factor delivery was developed for therapeutic angiogenesis.[129] Different release kinetics were observed for VEGF-165 and PDGF-BB delivered from a single PLGA scaffold; implantation of this

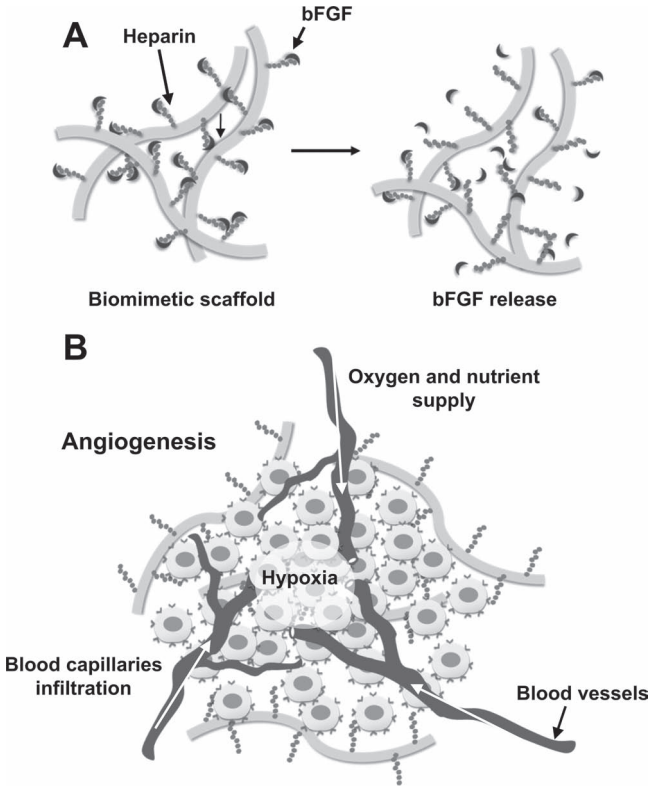

Figure 3. Bioactivation of a biomimetic scaffold with bFGF. A) A schematic illustration of bFGF binding and release on the heparinized scaffold. B) Controlled release of the angiogenic growth factor induces infiltration of blood vessels, allowing long-term survival of an implanted tissue.

scaffold induced the rapid formation of a mature vascular network in a rat model.

3.2.3. Composite Scaffolds Mimicking the Physiological Environment

Cells reside within the complex microenvironment of the ECM that contains many biomolecules including proteins, GAGs, and PGs. The complex structure and composition of the ECM dictates the mechanical properties of the matrix and cell-ECM interactions; scaffolds should therefore present chemical and physiological cues similar to those of the ECM to guide cell growth and function. Composite scaffolds have been prepared by physically combining materials with different chemical, mechanical, biological, and electrical properties.

Synthetic biodegradable polymers have been used in a variety of tissue engineering applications by blending them with natural polymers such as chitosan,[130] gelatin,[131] fibrin,[132] small intestinal submucosa (SIS),[133] hyaluronic acid,[132] and collagen[134] to improve biocompatibility for a specific target tissue. Collagen or gelatin blended with PCL was electrospun to fabricate fibers with a wide range of diameters from the micro- to nanoscale, which were used as substrates to culture several cell types.[135] These fibers enhanced osteogenic differentiation of hMSCs and pre-osteoblasts,[134] epidermal differentiation of keratinocytes,[136] and the formation of self-aligned skeletal myotubes of human primary skeletal muscle cells.[137] These results indicate that ECM analogue components are necessary to modulate a specific phenotype in an engineered tissue. An additional study demonstrated that type II collagen, one of the major maker proteins actively implicated in chondrogenic differentiation, and chondrointin sulfate incorporated in a PCL matrix markedly enhanced the secretion of collagen and GAGs from chondrocytes.[138] Absorption of a large amount of water is critical for lacuna formation from matrix-secreting chondrocytes and mechanical lubrication for cartilage tissue engineering, and therefore chitosan or hyaluronic acid have been incorporated into synthetic polymer matrices.[79,132] A composite of PCL core and collagen sheath was fabricated as a core-sheath fibrous structure, and human adipose-derived stem cells became more osteogenic on the fibers.[139] The improved cell compatibility, tailored mechanical properties, and degradation behavior of composite scaffolds have been used to stimulate the regeneration of many tissue types including bone, anterior cruciate ligament, blood vessel, trachea, and sciatic nerve with successful outcomes in animal models.[140,141]

Electrically conductive materials are promising candidate materials for controlling the function of electrically responsive cells such as neural and muscle cells, in which signal transduction is mediated by changes in membrane action potential.[142–144] A conducting substrate may be directly involved in electron transfer across the cell membrane or be utilized to convey external electrical stimuli. Polypyrrole and polyaniline are the most popular conducting polymers used in scaffolds and have been blended together with natural or synthetic polymers. Scaffolds containing gelatin and polyaniline demonstrated improved electrical conductivity and compatibility with H9c2 cardiac myoblast cells.[145] Recently, composite nanofibers based on PLCL and polyaniline were developed and used to culture C2C12 myoblasts; myogenic differentiation was significantly enhanced without external electrical stimulation.[146] Another group used electrospun nanofibers from composites of polyaniline with PCL and gelatin to demonstrate that the combination of conductive nanofibers and electrical stimulation enhanced neurite outgrowth.[147] In these studies, the amount of polyaniline blended with the counterpart matrix polymer was relatively small and therefore there were negligible adverse effects from the polyaniline.[148] Polypyrrole, which is another type of conducting polymer, has been used as an additive[149] or to coat[150] conventional synthetic degradable polymers mainly for neural and muscle tissue regeneration as well as to stimulate other types of cells such as fibroblasts.[151] Primary myoblasts cultured on a mixture of ECM components with polypyrroles achieved different stages of myogenic differentiation.[152] Furthermore, polypyrroles have been fabricated as micropatterned[153] or conductive core-sheath[154] nanofibrous structures, which emphasize the importance of both the electrical and topographical characteristics of scaffolds for modulation of the function of neural cells. These studies demonstrate that incorporation of electrical conductivity into tissue engineering scaffolds can have a substantial effect on cell behavior.

Composite scaffolds have been extensively studied in the area of formation of structural tissues such as bone, which has a high compressive strength and unique chemical

composition.[140] The high strength of scaffolds required for bone tissue engineering is obtained by incorporating rigid reinforcing particles such as hydroxyapatite, calcium carbonate, beta-tricalcium phosphate, and/or calcium phosphate[155] into synthetic or natural polymer matrices. In particular, natural polymers including gelatin, collagen, chitosan, and elastin usually show poor mechanical properties due to their hydrophilic nature, while incorporation of the above-mentioned particles significantly improves the strength and stiffness of the resulting scaffolds. Optimization of particle composition is critical to obtain the appropriate mechanical properties and recently, nanosized particles as compared to microscopic ones have been shown to greatly increase the compressive strength of scaffolds. For example, incorporation of modified single-walled carbon nanotubes (0.1 wt%) increased both the compressive strength and flexural modulus of poly(propylene fumarate)(PPF) scaffolds.[156] Ceramic particles, besides their mechanical strength, have the additional advantage of osteoconductivity because bone develops from highly organized collagen fibrous bundles, followed by mineralization. In addition, the major chemical constituents of ceramic particles are calcium and phosphate ions. The similarity of the chemical structure of hydroxyapatite, and other calcium and phosphate donor particles to that of bone facilitates the formation of bone when these materials are included in composites with other organic materials. For example, composites of hydroxyapatite and PLGA enhanced the osteogenic differentiation of human mesenchymal stem cells (hMSCs),[157] and nanofibers of tricalcium phosphates and PLLA enhanced the proliferation and alkaline phosphatase activity of adipose-derived stem cells (ADSCs), indicating that these particles are effective in controlling the osteogenic differentiation of progenitors.[158] These composites resulted in a significant improvement in bone growth in several in vivo implantation models; fibrin gels with hydroxyapatite and tricalcium phosphate or biphasic calcium phosphate (BCP) stimulated ectopic bone formation in muscle tissue in a sheep model for 6 months after implantation,[159] and composites of PLLA with demineralized bone powders accelerated bone formation in rats with critical-sized cranial defects.[160]

3.3. Scaffolds with a Biomimetic Structure

3.3.1. Scaffolds with Nano/Microscale Geometries

Cell behavior is modulated by numerous biochemical cues provided by biomacromolecules in the ECM. These biomacromolecules are assembled into 3D fibrous networks with finely controlled micro- or nanoscaled topographic patterns. An increasing body of experimental evidence suggests that cells can recognize various topographical structures with a diverse range of sizes through a phenomenon known as contact guidance, which can regulate cytoskeletal organization, cell shape, migration, and differentiation.[12,161–164] Therefore, scaffolds with defined topological patterns have been studied based on the hypothesis that topographic patterns can be used as instructive cues to direct cellular responses.

Despite recent advances in nanofabrication technology, surface patterns less than several hundred nanometers in scale have generally been limited to 2D substrates. Studies with nanograting geometry revealed that most cells, including endothelial cells,[165] fibroblasts,[166] and stem cells,[167,168] spread out and elongated along the grating axis. Cytoskeletal organization and focal adhesion assembly occurred in the same direction. Of particular interest are the responses of several tissue types with an aligned morphology under native conditions, e.g., muscle tissue (**Figure 4**).[169] A recent study showed that rat ventricular myocytes responded to nanogrooves with a width of

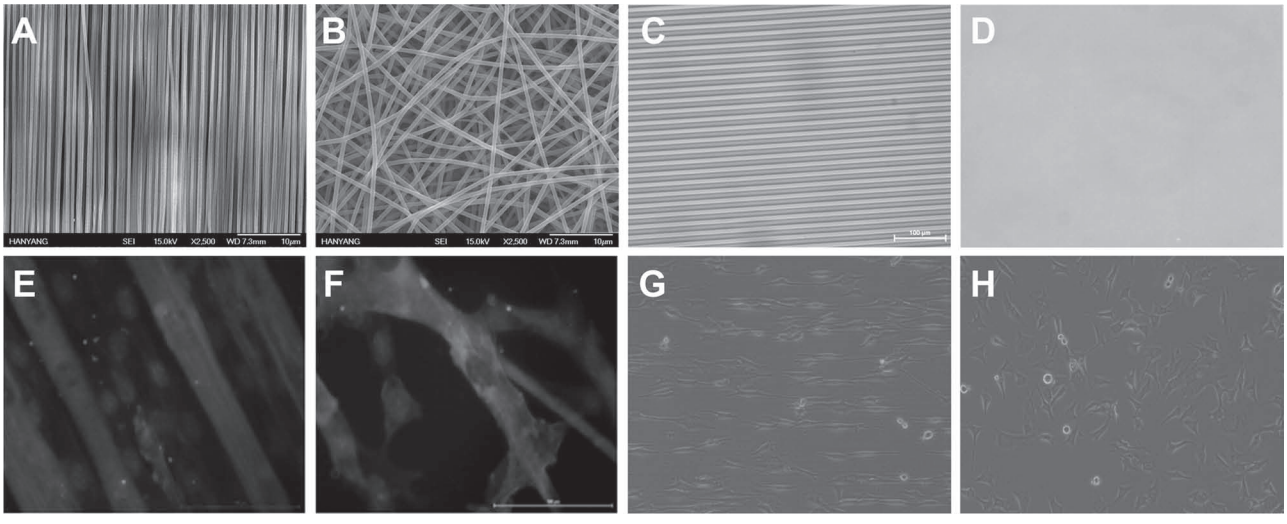

Figure 4. Control of cellular phonotype by culture of cells on tissue engineering scaffolds with micro/nano scaled geometry. Scanning electron microscopy (SEM) images of nanofibers with A) isotropic or B) anisotropic arrangement. Phase contrast images of hydrogels micropatterned with C) grooves of 25 μm and ridges of 10 μm, or without (D) any topological features. Sarcomeric myosin staining (green) and F-actin (red) (counterstaining for nuclei as blue) results for C2C12 myoblasts cultured on nanofibers with E) isotropic or F) anisotropic alignment. G,H) Phase contrast images of C2C12 myoblasts cultured on cell-interactive hydrogels of (C) and (D), respectively.

400, 600, or 800 nm, and that cell geometry, action potential, conduction velocity, and the expression of connexin 43 differed substantially according to the pattern size.[170] These results indicate that recapitulation of the native nano-scaled structure and geometry is imperative for successful tissue engineering. Other studies have emphasized the effects of nanometer pore sizes on the differentiation of cells. The osteogenic differentiation of rat bone marrow MSCs[169,171] and the differentiation of endothelial cells[172] cultured on TiO_2 nanotubes were enhanced on nanotubes with a diameter of 15 nm as compared to larger diameter nanotubes, and primary hippocampal neurons cultured on aluminum oxide substrates with diverse patterns showed faster neurite extension on surfaces with a 400 nm pitch as compared to surfaces with a 60 nm pitch,[173] indicating that different cell types may have different sensitivities to nanopatterns within a narrow window. In addition to size, the shape and arrangement of nanopatterns also affect cell behavior. For example, osteogenic differentiation of human MSCs can be regulated solely by diverse placements of 120 nm diameter pits in the form of a hexagon, square, displaced square, or random shape, even without any soluble stimulatory supplements.[174] In addition, ZnO substrates with nanorod[175] or nanoflower[176] patterns differentially affected the adhesion of fibroblasts and the osteogenic differentiation of MC3T3-E1 cells (mouse preosteoblastic cell line), respectively.

From a tissue engineering perspective, emulating the patterns of cells in a tissue at a micrometer scale is important because this may cause cells to undergo morphological changes necessary to achieve the 3D topology of the target tissue.[177] As mentioned in a previous section, the electrospinning technique allows fabrication of a 3D fibrous structure with isotropic or anisotropic patterns by simple alteration of solution viscosity or the velocity of the collecting drum.[178–180] The size of the fibers produced ranges from several hundred nanometers to a few micrometers. Particularly, fibers with a specific orientation have been shown to accelerate the induction of fibroblast migration and regulate the differentiation of human coronary artery smooth- and skeletal muscle cells.[181–183] In a recent study, Dang et al. obtained a monolayer of preferentially differentiated myoblasts derived from hMSCs in the absence of myogenic supplements by using aligned fibers prepared from hydrocybutyl chitosan.[184] The monolayer was later easily detached from the fiber while maintaining its structural integrity by exploiting the thermally responsive property of the synthetic polymers. Nanofiber scaffolds were also used to produce a tendon-to-bone interface by using a continuously graded, bone-like calcium phosphate coating.[185] High volume scaffolds with precise control over the structure at a micrometer scale can be prepared by solid free-form fabrication techniques.[186] Biodegradable polymers such as poly(propylene fumarate) (PPF),[187] poly(ε-caprolactone),[188] or poly(D,L-lactide)[189] have been used to prepare scaffolds with controllable porosity and various pore diameters by stereolithography.[190,191] These scaffolds usually support cell adhesion and proliferation, and most importantly, the optimum pore structure can be determined. Recently, the complex structure of the ventricular myocardium was mimicked by creating an accordion-like honeycomb microstructure from poly(glycerol sebacate).[192] The authors of that study showed that elongation was different depending on whether the scaffold was stretched in a longitudinal or circumferential direction,[192] indicating that the scaffold has direction-dependent mechanical properties similar to those of the heart. Collectively, these results suggest that scaffolds with a defined micro/nanostructure may induce cells to closely mimic the function of the native tissue.

3.3.2. Cell Sheet Engineering

One of the most important roles of tissue engineering scaffolds is to provide 3D structural integrity to direct transplanted or recruited cells to form assembled 3D tissue constructs. However, biomaterial-based scaffolds are unable to completely reconstitute native microenvironments for cells, which often cause implant failure, rejection, or unexpected cytotoxic problems. As an alternative to the use of scaffolds, delivery of scaffold-free cell sheets has been investigated over the last decade.[193,194] In principle, cell sheets can be generated from physical detachment of confluently expanded cell layers without the use of proteolytic enzymes. The resulting cell sheets maintain the complex microenvironment formed during the monolayer culture; i.e., cell–cell, cell–ECM, and ECM–ECM interactions remain intact. The driving force to detach cell sheets from the substrate can be a magnetic force,[195] electrostatic interactions provided by a polyelectrolyte surface,[196] or transformation of hydrophobic/hydrophilic properties on the substrate using temperature-responsive polymers or polypeptides with lower critical solution temperature (LCST) behavior.[197–200] Poly(*N*-isopropylacrylamide) (PNIPAAm) or elastin-like polypeptides (ELPs) with thermal responsiveness were physically or chemically grafted or coated onto normal cell culture dishes, and cells were cultured at 37 °C above the LCST of these materials and, consequently, the confluent cell sheet could be removed by simply changing the temperature to below the LCST of these materials. Cell sheets produced from various cell types based on this technique have been applied to several tissue defect models including heart,[201] cornea,[202] esophagus,[203] skin,[204] pancreas,[205] and periodontal ligament.[206] Although the initial work was limited to generation of a monolayer of cells, recent approaches combining photolithography and micro/nanofabrication process have demonstrated that it is possible to form 3D complex structures with multiple cellular populations.[207] For example, fibroblasts (FBs) were cultured as a monolayer and transferred to a gelatin-coated manipulator. In another dish, endothelial cells (ECs) were cultured on the micropatterned PNIPAAm surface to accelerate the formation of a vascular tubular structure, which was transferred to the FB monolayer. Multiple layered cell sheets composed of alternating fibroblasts and patterned endothelial cells were obtained by repeating these steps. An additional approach involved the grafting of PNIAAm on textured polystyrene dishes fabricated by hot-embossing or electron-beam lithography, resulting in the creation of a 3D structure similar to that of native organs.[208,209]

4. In Situ Gelled Peptide Hydrogel Scaffolds

While numerous engineering approaches have been developed to create synthetic polymer-based, pre-fabricated scaffolds,

new classes of bioinspired materials, peptide-based biomaterials have excited great interest.[210–213] These biomaterials are used to create in situ gelled, biomimetic hydrogel scaffolds for tissue repair and reconstitution, as well as controlled drug release reservoirs.[214,215] Rationally designed peptides of high or low molecular weight are derived from native proteins and rapidly assemble into hydrogel scaffolds in vivo by physical or chemical crosslinking mechanisms, providing encapsulated cells with an artificial ECM environment. Peptides with environmental stimuli-responsiveness are physically self-assembled by controlling the temperature, pH, or ionic strength while peptides with reactive functional groups are in situ chemically crosslinked by using multi-arm crosslinkers, enzymes, radical- or photopolymerization, and gamma irradiation.[213–218] Using these physical or chemical means, a liquid-like precursor solutions can be easily mixed with target cells, administered into the potential tissue defect site, and then gelled within several minutes to create a biomimetic scaffolds; this is non-invasive compared to the implantation of prefabricated scaffolds with cells. In particular, ECM-derived polypeptides including elastin-based peptides, collagen-like proteins, as well as fibrins and spider silk proteins have been widely used in place of synthetic biodegradable polymers to synthesize biomimetic scaffolds because they show good biocompatibility with low cytotoxicity, minimal immune responses and inflammation, have controllable degradation rates in vivo, and can be broken down into natural amino acids that can be catabolized or anabolized in the body.[219,220] In this section, we review two main classes of in situ gelled peptide hydrogel scaffolds formed by different gelling mechanisms: 1) self-assembling peptide hydrogel scaffolds stimulated to assemble by external stimuli and 2) in situ chemically crosslinked peptide hydrogel scaffolds that incorporate rationally designed, biologically inspired peptide-based materials prepared via genetic and protein engineering or chemical synthesis. We focus in particular on the incorporation of bioactive peptides into peptide hydrogel scaffolds to serve as biological cues for different cell-to-biomaterial interactions.

4.1. Peptide Hydrogel Scaffolds by Self-Assembly

The primary interest has been given to the hydrogel system based on rationally designed peptides that can be environmentally responsive and self-assemble into complex 3D networks. External stimuli often trigger conformational changes of peptides. The main building blocks of peptide-based hydrogels can be prepared by either genetic engineering or chemical synthesis. Especially, advances in genetic and protein engineering tools have facilitated the development of genetically engineered polypeptides with amino acid sequences derived from native proteins that serve as biomimetic scaffolds. The main advantages of genetically encoded polypeptides for tissue engineering are as follows: 1) genetically encoded polypeptides have amino acid sequences with defined physicochemical properties, for example, stimuli-responsiveness can be precisely controlled, and specific amino acid sequences for structural, chemical, or biological cues can be freely introduced at any position in the polypeptide chain by genetic engineering; 2) they yield monodisperse polypeptides with molecular weights in the wide range from several kDa to ≈200 kDa; 3) they can be readily expressed at several hundred milligrams per L in a shaker culture by using bacterial expression systems; and 4) enzymatically cleavable sequences can be introduced to control the rate of protease-mediated degradation of the hydrogel scaffold in vivo.[210–212,221–224] In this section, we provide an overview of self-assembling peptide hydrogel scaffolds based on genetically engineered peptides (elastin-based peptides, elastin-based peptide block copolymers, tropoelastin-based peptides, and coiled-coil peptides) and chemically synthesized peptides (β-sheet forming oliogopeptides, β-hairpin peptides, and peptide-amphiphiles).

4.1.1. Genetically Engineered Peptide Hydrogel Scaffolds

Elastin-Based Peptides: Elastin-based peptides (EBPs) including elastin-like polypeptides, elastin-mimetic polypeptides, and tropoelastin-based polypeptides are of great interest as biomimetic scaffolds in the field of tissue engineering because they are derived from elastin, which is one of the major ECM proteins.[210–213] Importantly, EBPs can undergo an inverse temperature phase transition with a lower critical solution temperature (LCST). They are completely soluble in aqueous solutions, but due to conformational changes as the temperature is raised above LCST, they shed hydrated water molecules bound to their polypeptide chains and become desolvated and insoluble in aqueous solutions, which is a unique biophysical property that enables a sol–gel transition and facilitates their use as injectable biomaterials. Therefore, EBPs have been widely investigated as components of biomimetic scaffolds for regeneration of cartilage, intervertebral disc, vascular graft, liver, and ocular tissue as well as cell sheet engineering.[210–213]

As one of the simplest EBPs, elastin-like polypeptides (ELPs) have the repetitive pentapeptide sequence [Val-Pro-Gly-Xaa-Gly]$_n$, where Xaa can be any amino acid other than Pro, and are derived from the hydrophobic domain of a tropoelastin composed of alternating hydrophobic domains and hydrophilic domains with crosslinked Lys sites.[225,226] In addition, as pointed out earlier, the peptide sequence of ELPs can be designed at the genetic level, so genetically encoded peptide domains that provide specific physical, chemical, or biological cues and that are resistant to protease degradation can be freely incorporated into ELP chains that can then form hydrogel networks.[224,227] Chilkoti, Setton, and co-workers pioneered a variety of cartilage tissue engineering applications by taking advantage of the elastic and gelatinous property of ELPs above the LCST to develop load-bearing, biomimetic peptide hydrogel scaffolds.[212,228] Thermally triggered coarcervates of ELPs are useful as in situ gelled hydrogels for cartilage tissue repair because they have a range of rheological and mechanical properties, and ELPs with the repetitive pentapeptide sequence of [Val-Pro-Gly-Val-Gly]$_n$ showed good biocompatibility with minimal cytotoxicity and immune response when implanted in vivo.[229–231] A concentrated solution of an aliphatic ELP became phase-separated into an aqueous phase and an ELP coacervate phase as the temperature increased above the LCST, and the complex shear modulus (G^*) of the ELP coacervate was 100 Pa, which is similar to that of collagen and hyaluronan.[229] Due to the structural integrity of the ELP coacervate above its LCST, its

half-life was 25-fold longer than soluble ELP when injected into a rat knee.[232] When chondrocytes were encapsulated and in vitro cultured for 2 weeks within the ELP coacervate above the LCST, they maintained round shape with synthesis of sulfated glycosaminoglycan (S-GAG) and type II collagen, characteristic of native cartilage ECM. Another study with human adipose-derived adult stem (*h*ADAS) cells encapsulated in the ELP coacervate demonstrated selective differentiation of *h*ADAS cells into chondrocytes without any exogenous chondrogenic supplements of TGF-β1 or dexamethasone under low oxygen tension conditions.[233] The hADAS cells cultured in the ELP coacervate showed up-regulated gene expression of both SOX9 and type II collagen, indicative of a cartilage phenotype, while type I collagen, which is characteristic of a fibroblast phenotype, was down-regulated.

Multifunctional ELP coacervates were also formed from ELP fusion proteins because the ELP fusion proteins were thermally coacervated due to a conformational change in the ELPs while the bioactivity of the fusion domain was retained.[234] For example, for intra-articular treatment of osteoarthritis, the ELP-interleukin-1 receptor antagonist (ELP-IL-1Ra) fusion protein formed stable ELP-IL-1Ra coacervates above its LCST in vitro and controlled release of the fusion protein from the coacervates was observed.[235] The ELP-IL-1Ra fusion protein functioned as a IL-1 receptor antagonist in human intervertebral disc cells; it inhibited IL-1-mediated lymphocyte and thymocyte proliferation as well as IL-1-induced tumor necrosis factor α (TNFα) expression and matrix metalloproteinase 3 (MMP-3) and ADAMTS-4 mRNA expression. A variety of functional ELP fusion proteins have been synthesized by genetic fusion of ELPs with bioactive peptides or protein domains: 1) a soluble tumor necrosis factor receptor II (sTNFRII) was used to sequester TNFα from its target receptor and attenuate inflammation; 2) an epidermal growth factor was used to enhance cell proliferation; 3) a c-myc oncogene inhibitor was used to antagonize the transcriptional activation and retard the growth of breast carcinoma cells; and 4) a penetratin, which is a cell penetrating peptide, was used to enhance the translocation of ELPs through the cell membrane.[221,236–239] Furthermore, ELPs have been interspersed with cell binding domains such as GRGDS sequences derived from fibronectin, laminin-derived IKVAV sequences, or collagen binding domains at predetermined positions to serve as an artificial ECM.[240–243] Fusion ECMs at temperatures above the LCST promoted endothelial cell adhesion and spreading in vitro, and were used for cell-sheet recovery by disaggregation of the ELP domains as the temperature was lowered below the LCST.

Elastin-Based Peptide Block Copolymers: Physically crosslinked, load-bearing peptide hydrogel scaffolds were developed from ABA-type EBP triblock copolymers by preventing phase separation during EBP aggregation and enhancing physical crosslinking between the aggregated EBPs.[213–215,244] For example, ELP triblock copolymers with elastomeric domains of $[Val\text{-}Pro\text{-}Gly\text{-}Xaa\text{-}Gly]_n$ were synthesized, which are composed of a hydrophilic middle-block with a high LCST above 90 °C and a hydrophobic end-block with a low LCST in the range of 25 °C to 37 °C. Both ELP end blocks underwent temperature-triggered aggregation above the low LCST, leading to physical crosslinking of the ELP aggregates due to inter- or intramolecular hydrophobic interactions, while the ELP middle block maintained its hydrated state. The concentrated solution of ELP triblock copolymers with an approximately equal block length ratio showed thermally reversible rheological- and mechanical behaviors, and had a gelation temperature where G' (elastic modulus) was equal to G'' (viscous modulus) in dynamic shear rheological testing as a function of temperature.[210] The gelation temperature is defined as the cross-over point of G' and G'', meaning that the solid-like aggregates of the ELP end blocks acted as physically crosslinked domains and the liquid-like, hydrated ELP middle blocks acted as flexible, hydrophilic domains, resulting in the formation of a physically crosslinked hydrogel network without any phase separation. Furthermore, the thermal transition temperatures and rheological and mechanical properties of the ELP triblock copolymers were largely determined by the amino acid sequence and charged state of the hydrophobic block and the salt concentration. This suggests that the load-bearing properties of ELP block copolymer hydrogels can be finely tuned by rationally designing ELP blocks and optimizing their ionic strength under physiological conditions (unpublished work).

Using a similar approach, EMP triblock copolymers composed of a plastic end block of $[Val\text{-}Pro\text{-}Ala\text{-}Val\text{-}Gly]_n$ and an elastomeric middle block of $[Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly]_n$ were synthesized for thermally triggered self-assembly.[213–215,245] The replacement of Gly with Ala in the third amino acid position of the repetitive pentapeptide unit converted its 3D structure from a type II β-turn structure of Pro-Gly residues to a type I β-turn structure of Pro-Ala residues, conferring plasticity to the EMP end blocks instead of elasticity.[214,215,245] In addition, the plastic end block was a hydrophobic block with a low LCST below 37 °C and the elastomeric middle block was a hydrophilic block with a high LCST above 75 °C. Due to the structural differences and hydrophobicity of the plastic end block, the EMP triblock copolymers formed a thermally triggered, physically crosslinked hydrogel scaffold as shown by the rheological measurements; the value of G' was 1–2 orders higher than that of G'' above the LCST, indicating that the copolymer had a solid-like viscoelasticity.[214,215] The rheological and mechanical properties of the EMP triblock hydrogels were largely controllable by using substantially larger plastic end blocks than the elastic middle block as well as charged or uncharged middle block. Notably, upon injection in vivo, the self-assembling EMP hydrogel scaffolds showed robust long-term biostability and biocompatibility for a period of 1 year without any significant inflammatory responses or calcification in the subcutaneous space and peritoneal cavity of a mouse model.[246–248] This suggests that physically crosslinked EMP hydrogel scaffolds without any chemical or ionic bonding have great potential as injectable biomimetic hydrogel scaffolds for tissue engineering.

In addition to this self-assembling property of the injectable biomaterials described above, various protein films composed of EMP triblock copolymers were formed by a solvent casting/evaporation process under different solvent environments including 2,2,2-trifluoroethanol (TFE), water, or their mixtures.[218,244] Depending on the solvent type and incubation temperature during solvent evaporation, the secondary structure of the EMP triblock copolymer changed, because TFE as a fluorinated alcohol formed strong solid-state complexes with

polyamides.[218,244] The TFE solvated both the hydrophobic end block and hydrophilic middle block promoting interpenetration and entanglement of both blocks but water solvated only the hydrophilic middle block except the aggregated hydrophobic end block above the LCST. This resulted in protein films with varied microstructures and mechanical properties when hydrated in an aqueous solution.[213,218,244,247,248] In this regard, controllable nano- and microscaled protein films containing biological drugs can be useful as drug-eluting implants or coating films on biomedical devices to finely tune their release kinetics. Protein nanofibers of EMP triblock copolymers were also prepared by an electrospinning technique under the different solvent conditions mentioned above.[217] Various tubular constructs were fabricated as nonwoven nanofiber scaffolds with different mechanical properties for vascular tissue engineering according to the nanofiber deposition process.[213,246,248]

Tropoelastin-Based Peptides: The human tropoelastin gene is composed of 36 exons encoding alternating hydrophobic and hydrophilic blocks of tropoelastin, which is the monomer of insoluble elastin. Keeley and co-workers developed recombinant human tropoelastin-like multiblock copolymers by introducing exons 20 and 24 as hydrophobic blocks and exons 21 and 23 as hydrophilic and crosslinkable blocks.[249] Tropoelastin-like tri-, penta-, and hepta-block copolymers with various molecular weights ranging from 10 kDa to 31 kDa showed similar inverse phase transition behavior to EBPs due to coarcervation of the hydrophobic blocks. Above the LCST, strong interactions between the coarcevated hydrophobic blocks made the multiblock copolymers self-assemble into fibrillar structures, which allows Lys residues in the hydrophilic, crosslinkable blocks to come closer together, facilitating further chemical linkages.[250–253] This self-assembling property was strongly affected by both hydrophilic blocks and hydrophobic blocks because 1) deletion of a flexible hinge region in the center of the hydrophilic blocks increased the α-helical content, promoting coarcervation and 2) TFE mixed with water changed the LCST behavior by affecting the secondary structure of the hydrophobic block. As an alternative to tropoelastin-like multiblock copolymers, Vrhovski and co-workers cloned the full-length recombinant human tropoelastin gene with 36 exons encoding alternating hydrophobic and hydrophilic segments.[254,255] The expressed human tropoelastin displayed LCST behavior, enabling strong interactions between tropoelastins and crosslinking of adjacent lysine residues within the hydrophilic blocks. Likewise, polypeptides encoded by exon 30 of human tropoelastin formed self-assembled coarcervates above the LCST, resulting in amyloid-like fibril structures due to cross β structures with antiparallel β sheets over the fibrils.[256–258] In addition to tropoelastin- and elastin-based polypeptides, genetically-encoded tropoelastin-silk or elastin-silk block copolymers have been synthesized and used as self-assembling, injectable polypeptide hydrogel scaffolds for cartilage tissue repair, differentiation of human mesenchymal stem cells, and drug- or gene-delivery reservoirs.[259–263] Furthermore, 3D biomimetic composite scaffolds composed of elastin or tropoelastin and biodegradable synthetic polymers including polycaprolactone, have been prepared for cartilage tissue regeneration and functional tissue engineering.[213,264,265]

Coiled-Coil Peptides: While the quest for biologically inspired materials such as elastin-, silk-, or collagen-like polypeptides for the development of biomimetic scaffolds continues, the use of the unique protein folding motif of coiled-coils to fabricate environmentally responsive, self-assembled polypeptide hydrogels has attracted great interest.[266,267] Coiled-coil proteins, for example, the leucine zipper motifs of Jun and Fos transcription factors, have a super-helical architecture composed of two or more α-helical structures intertwined around each other via non-covalent interactions (van der Waals interactions and ionic interactions) between their side chains. The primary sequence of each helical unit of the coiled coil proteins is composed of heptad repeats designated $(abcdefg)_n$, where 1) *a* and *d* are hydrophobic amino acids such as Leu, 2) *e* and *g* are charged amino acids, particularly Glu, and 3) *n* is the number of heptad repeats. The coiled-coil proteins are self-assembled into hydrogels by strong hydrophobic interactions between *a* and *d* residues positioned on a single face of the helical chains and are stabilized by salt bridges between charged *e* and *g* residues positioned on the other face of the helical chains. In particular, environmental stimuli such as pH, temperature, and ionic strength largely affect the self-assembly of coiled-coil structures because of different degrees of intermolecular interactions between the helical chains.[266,267] For example, the melting temperature of coiled-coil dimers becomes low with increasing pH due to increased repulsive electrostatic interactions between the negative charges of deprotonated Glu residues.[268,269] As proof of concept, Tirrell and co-workers developed pH- and temperature responsive, self-assembling artificial proteins by introducing leucine zipper protein folding motifs.[270,271] They rationally designed genetically encoded, ABA-type triblock peptides consisting of leucine zippers at the A end blocks for inter- and intra-chain interactions, and alanylglycine-rich polyelectrolyte middle blocks as B middle blocks for hydration due to their random coil structure.[270–273] The triblock proteins in situ gelled under physiological conditions by forming dimeric and tetrameric aggregates of terminal leucine zippers, and these self-assembling protein hydrogels could be disintegrated by changing the temperature or pH. Furthermore, to control the erosion rate of ABA-type triblock hydrogels with leucine zippers, a new class of ABC-type triblock proteins was genetically synthesized by introducing an identical random coil B middle block and two dissimilar coiled-coil end blocks that are unable to associate with each other as A and C blocks.[273] Due to minimized intrachain interactions between the A and C blocks and enhanced interchain interactions between A and A blocks and C and C blocks, the degradation of ABC-type protein hydrogels was reduced by 2–3 orders of magnitude compared to ABA- and CBC-type triblock protein hydrogels. More recently, biohybrid materials, namely 1) poly(*N*-(2-hydroxypropyl)methacrylamide)-based hydrogels with the coiled-coil domain of the motor protein kinesin or VSSLESK heptad repeats and 2) polyethyleneglycol-based hydrogels with the coiled-coil domains of human fibrins were self-assembled through strong molecular interactions between the coiled-coil domains as physical crosslinkers.[266,267,274,275] These precisely controlled protein-based biohybrid hydrogels can potentially be used as controlled drug delivery reservoirs and biomimetic hydrogel scaffolds for 3D cell culture and regenerative medicine.

4.1.2. Chemically Synthesized Peptide Hydrogel Scaffolds

Compared with high molecular weight genetically engineered polypeptides, low molecular weight chemically synthesized oligopeptides have been intensively developed to create self-assembling hydrogel scaffolds. Pioneering classes of self-assembling synthetic peptides including β-sheet-forming oligopeptides, de novo designed β-hairpin peptides, and peptide-amphiphiles have been explored and are reviewed in this section.

β-Sheet-Forming Oligopeptides: The first ionic oligopeptide, AEAEAKAKAEAEAKAK (EAK16-II), which forms a β-sheet structure under the physiological conditions of a neutral pH and optimized salt concentrations, was derived from a left-handed Z-DNA binding protein in yeast.[276–278] Ionic oligopeptides generally have 16 amino acids composed of periodic repeats of alternating charged, hydrophilic amino acids (K or R as positively charged amino acids and D or E as negatively charged amino acids) and aliphatic, hydrophobic amino acids (A, V, I, L, Y, P, or W as hydrophobic amino acids), and these ionic oligopeptides spontaneously assemble into nanofiber-based hydrogel scaffolds. The dynamic self-assembly rates of these peptides as well as the rheological and mechanical properties of the scaffolds are largely controlled by non-covalent interactions among the β-sheet structures, which have an amphiphilic character; there are 1) strong ionic interactions between alternating positive and negative charges on one side of the sheet, 2) staggered hydrophobic and van de Waals interactions among hydrophobic residues on the other side of the sheet, and 3) hydrogen bonding interactions between the sheet backbones. In addition, depending on ionic strength, pH, and temperature, the β-sheet structures undergo conformational changes that determine their self-assembling characteristics. Recently, chiral oligopeptides made of all D-amino acids have been shown to form nanofiber scaffolds and resist proteolysis by enzymes with natural L-amino acids when implanted in vivo. A series of self-assembling nanofiber scaffolds have been shown to be excellent 3D matrices for tissue engineering because they supported the attachment and growth of endothelial cells, neuronal cells, hepatocytes, chondrocytes, and osteoblasts.[279–283] Furthermore, various biologically active motifs have been introduced at the end of the oligopeptides for cartilage and bone tissue engineering; osteogenic growth peptide ALK (ALKRQGRTLYGF), osteopontin cell adhesion peptide DGR (DGRGDSVAYG), and the RGD cell binding peptide PGR (PRGDSGYRGDS).[284–288] When the functionalized oligopeptides were mixed with the original β-sheet-forming oligopeptides at varied mixing ratios, nanofiber scaffolds formed spontaneously, and these scaffolds supported osteoblast proliferation, differentiation, and 3D migration.[284,286,288,289]

In contrast, another β-sheet forming short peptide, QQKFQFQFEQQ (Q11), has been shown to act as an immune adjuvant when the short epitope moiety encoded by the 17 amino acids of ovalbumin is incorporated at the end of the Q11 peptide.[289,290] As shown in **Figure 5**, epitope-modified Q11-based peptides are self-assembled into β-sheet fibrillar scaffolds that elicit high antibody titers without any adjuvant due to exposure of bioactive epitope moieties on the surfaces of the scaffolds. These results suggest that β-sheet forming peptides containing functional motifs or epitope moieties are promising 3D biomimetic hydrogel scaffolds for tissue regeneration or antibody production against distinct peptide epitopes in vivo.[95,291,292]

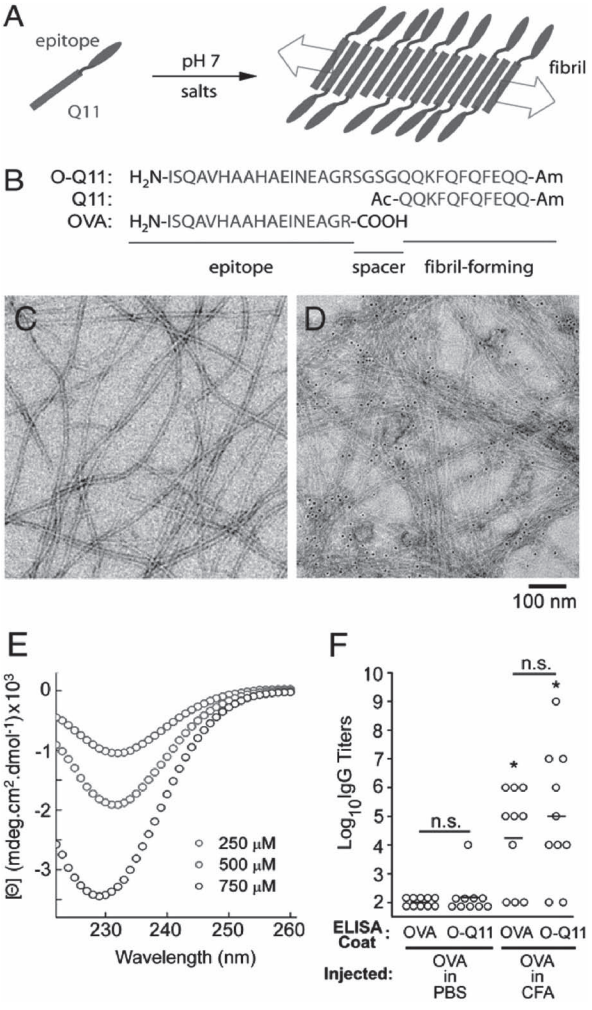

Figure 5. A) Schematic and B) amino acid sequences of epitope incorporated, self-assembling peptides as immune adjuvants. C,D) Transmission electron microscopy (TEM) images, E) circular dichroism profiles, and F) ELISA-based immunoglobulin G (IgG) quantification of the epitope-exposing β-sheet fibrils. C) With a Q11 sequence self-assembling into β-sheet fibrils, the O-Q11 peptide, an ovalbumin (OVA) fragment-SGSG spacer-Q11 peptide, also spontaneously forms β-sheet fibrils exposing the OVA epitopes. D) Biotinylated O-Q11 peptides at the N-terminal self-assemble into fibrous structures, and streptavidin-gold nanoparticles are bound to the surface of the nanofibers due to strong molecular interactions between biotin and streptavidin, indicating that the OVA epitopes at the N-terminals are surface-exposed. E) The O-Q11 peptides form nanofibrils with a high degree of β-sheet or β-turn structure, as determined by circular dichroism measurements. F) An ELISA plate coated with OVA and O-Q11 peptides shows similar titers of OVA-reactive IgG when incubated with antisera from mice immunized with OVA, either with or without complete Freund's adjuvant (CFA). Each point and bar in (F) represents one mouse serum and the mean of the titer of OVA-reactive IgG, respectively. The n.s. in (F), not statistically different. Reproduced with permission.[290] Copyright 2010, National Academy of Science.

De novo Designed β-Hairpin-Forming Peptides: Compared with β-sheet-forming peptides, de novo designed β-hairpin-forming

peptides with 20 amino acid residues have strong β-turn forming tetrapeptides, -V^{D}P^{L}PT- (^{D}P^{L}P: d-form Pro and l-form Pro) flanked by alternating VK sequences at both ends for β-sheet formation.[293–295] For example, the short amphiphilic peptide, VKVKVKVK-V^{D}P^{L}PT-KVKVKVKV-NH_2 (MAX1), folds into a β-hairpin structure, followed by intermolecular associations between the β-hairpins via the formation of lateral hydrogen bonds between the folded hairpins as well as facial hydrophobic interactions between the Val-rich faces of the hairpins, resulting in self-assembled hydrogels rich in β-sheets.[296,297] Intramolecular β-hairpin folding-induced self-assembly can be controlled by various stimuli including pH, temperature, and salt, as follows: 1) basic pH condition strongly induces the formation of β-hairpins; if the pH is below the pK_a of Lys, there is intrastrand charge–charge repulsion between Lys residues, resulting in unfolding of β-hairpins; 2) increasing temperature induces the dehydration of the hydrophobic Val or Thr residues of the peptides, resulting in hydrophobic collapse-induced folding of β-hairpins; and 3) raising the ionic strength decreases charge–charge repulsion between Lys residues by screening their charges, inducing intramolecular β-hairpin folding.[296,298,299] Recently, light- or zinc-triggered intramolecular β-hairpin folding for self-assembly was developed by introducing an α-carboxy-2-nitrobenzyl photocage or zinc-binding non-natural amino acid into the peptide.[295,300] This peptide remains unfolded due to the negatively charged state of the photocage or the zinc-binding amino acid at a neutral to basic pH. However, UV light induces photolysis of the photocage or chelation of the zinc to a non-natural ligand, thereby triggering intramolecular β-hairpin folding. A series of these self-assembling hydrogels showed a mechanically rigid property with a storage modulus (G') of 0.1–3 kPa under various buffer conditions. When fibroblasts were embedded within the 3D hydrogel scaffolds, the scaffolds supported the adhesion, migration, and proliferation of fibroblasts without any severe cytotoxicity.[301,302] Furthermore, β-hairpin peptide hydrogels are shear-thinned, which allows homogeneously encapsulated mesenchymal stem cells within the hydrogels to be injected via syringe.[303,304] This injectable cell delivery system was also used as a drug delivery reservoir by 3D encapsulation of curcumin in the hydrogels.[305] In addition, biodegradation of the β-hairpin peptide hydrogels by proteolysis with matrix metalloproteinase-13 (MMP-13) was controlled by incorporating a recognition peptide sequence of MMP-13 in the peptides.[306]

Peptide Amphiphiles: While various self-assembling oligopeptides have been developed as described above, a hydrophilic peptide head covalently bonded to a hydrophobic alkyl tail containing 6–22 carbon atoms, referred to as a peptide amphiphile (PA), was found to self-assemble in aqueous solution into supramolecular nanofibers. In general, PAs are charged molecules so that screening their ions by controlling pH and ionic strength triggers molecular self-assembly into 3D fibrous hydrogel scaffolds.[307] PA nanofibers can be further stabilized by oxidation of three Cys residues introduced next to the hydrophilic Gly residues of the PAs. In a recent study, a series of bioactive and hydrophilic peptides were introduced at the end of the PAs, which upon self-assembly become highly exposed on the surfaces of the PA nanofibers.[308,309] To be more specific, PAs with 1) RGD sequences for cell adhesion or the laminin epitope IKVAV for promoting and directing neurite growth,[310] 2) HSNGLPL binding epitopes for transforming growth factor-β1 (TGF-β1) to promote chondrogenic differentiation of human mesenchymal stem cells and regeneration of articular cartilage by controlled release of TGF-β1 from the TGF-β1-bound PA nanofibers,[311] 3) membrane-disrupting peptides $(KLAKLAK)_2$ for induction of selective breast cancer cell death,[312] and 4) a single phosphorylated Ser residue for strong interaction with calcium ions and induction of hydroxyapatite mineralization were synthesized and self-assembled.[313] The biological epitope densities on the PA nanofibers were controlled by co-assembling bioactive PAs with the original PAs so that the epitopes on the PA nanofibers had maximum binding affinity to specific proteins and cells or maximum bioactivity. Furthermore, PA nanofibers have been shown to be useful as therapeutic protein delivery reservoirs because controlled release of sonic hedgehog protein or angiogenic factors such as vascular endothelial growth factor and basic fibroblast growth factor encapsulated within the PA nanofiber hydrogels promoted regeneration of cavernous nerves and sprouting of islet endothelial cells from the islets, respectively.[308,309,311,314]

4.2. In Situ Chemically Crosslinked Peptide Hydrogel Scaffolds

Although self-assembling peptide hydrogel scaffolds with physical crosslinking have numerous advantages over in situ chemically crosslinked counterparts, including easy processability and avoidance of removal of unreacted reagents or byproducts after chemical crosslinking for in vivo applications, rapid chemical crosslinking of peptides has been studied intensively because it can be used to develop injectable peptide hydrogel scaffolds with controllable chemical crosslinking density, structural integrity, and load-bearing mechanical properties.[210–212,222] However, few studies of in situ gelation of peptides by chemical crosslinking in the context of tissue engineering have been performed because of 1) the slow reaction rate of chemical crosslinking reactions in aqueous solution and 2) the cytotoxicity and lack of biocompatibility of crosslinking reagents or byproducts produced from chemical reactions. In this section, considering these matters, we review in situ chemically crosslinked elastin-based peptide hydrogel scaffolds for load-bearing cartilage and intervertebral disc tissue engineering applications as well as chemically crosslinked, prefabricated scaffolds for vascular graft tissue engineering. Additionally, we briefly review 3D patterned, chemically crosslinked peptide hydrogel scaffolds, which are responsive to enzymatic activity.

4.2.1. Chemically Crosslinked Elastin-Based Peptide Hydrogel Scaffolds

In situ chemically crosslinked ELP hydrogel scaffolds have been used for load-bearing cartilage tissue repairs, including defect filling and cartilage regeneration.[212] It was recently reported that the Mannich-type condensation reaction of amine reactive hydroxymethyl-phosphines (>P–CH_2–OH) with primary and secondary amines of proteins was suitable for rapid and biocompatible chemical crosslinking of thermally-responsive ELPs in an aqueous solution; water was the only byproduct and chem-

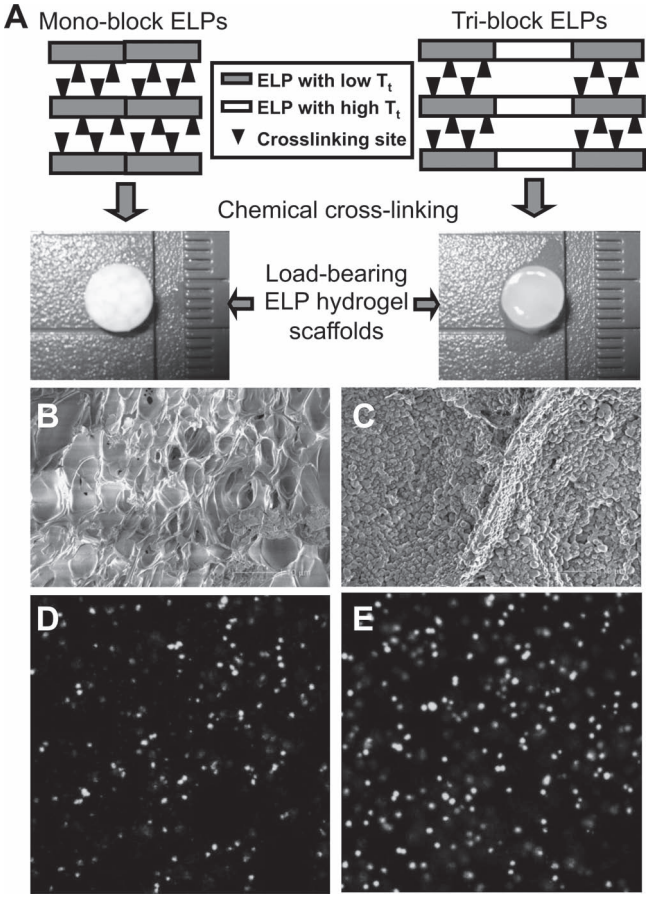

Figure 6. A) Schematics of ELP block copolymer architectures and photographs of in situ chemically crosslinked ELP hydrogel scaffolds. B,C) Cross-sectional SEM images of freeze-fractured ELP hydrogel scaffolds and D,E) fluorescent cell images at day 3 showing cell survival of fibroblasts encapsulated in the ELP hydrogel scaffolds. B,D) Monoblock ELP hydrogel scaffold and C,E) triblock ELP hydrogel scaffold. Scale bars are 10 μm in (B) and (C). Cell survival of encapsulated fibroblasts was determined using a fluorescent cell viability/cytotoxicity assay. Reproduced with permission.[316] Copyright 2008, American Chemical Society.

ically stable aminomethylphosphine (>P–CH_2–N<) linkages were formed.[315–317] As shown in **Figure 6**a series of ELP block copolymers composed of a cross-linkable, hydrophobic block with charged Lys residues and an aliphatic, hydrophilic block with various ELP block length ratios were rapidly crosslinked with a biologically benign organophosphorous crosslinker, *β*-[tris(hydroxymethyl)-phosphino]-propionic acid (THPP) and gelled in-situ within 5 minutes, while inter- or intramolecular chemical networks evolved in an hour with equilibrated rheological and mechanical properties due to increased chemical crosslinking densities and aggregation of the ELP hydrophobic blocks. The THPP-crosslinked ELP block copolymer hydrogels had different porous microstructures with various chamber diameters and distributions depending on the block copolymer architecture. In addition, their rheological and mechanical properties were largely controlled by different crosslinkable Lys densities in the hydrophobic block as well as varied lengths of the hydrophilic middle block. The THPP-crosslinked ELP hydrogels by themselves were used as injectable 3D scaffolds in vivo for filling in an osteochondral defect in a goat model and were found to be load-bearing and promote cartilage regeneration with a newly synthesized cartilage-like matrix.[228,318] Likewise, primary chondrocyte-encapsulated ELP scaffolds promoted cartilage matrix synthesis and regeneration in vitro. Chemical crosslinking density and ELP concentration are important determinants of the load-bearing capacity of scaffolds for cartilage tissue engineering applications.

Compared with in situ gelled ELP hydrogel scaffolds, a number of chemically crosslinked, prefabricated hydrogel scaffolds of ELPs, multiblocks of ELP fusion proteins with integrin binding domains, and ELP derivatives for cartilage and intervertebral disc tissue repair, vascular grafts, and stem cell sheets have been developed by using multi-arm chemical crosslinkers such as tris-succinimidyl aminotriacetate (TSAT), bis(sulfosuccinimidyl) suberate, glutaraldehyde, hexamethylene diisocyanate, genepin, tissue transglutaminase, radical- or photo-polymerization, gamma irradiation, or [3 + 2] Huisgen cycloaddition.[213,215–217,240–242,319–323] For example, Lys-incorporated ELPs in dimethylsulfoxide and dimethylformamide were chemically crosslinked by TSAT and formed homogeneously crosslinked ELP hydrogel scaffolds with mechanical properties that were largely determined by the frequency of lysine residues and the concentration and molecular weight of the ELPs.[323] Therefore, chemically crosslinked elastin-based hydrogel scaffolds possessing a wide range of load-bearing properties are useful for cartilage tissue repair when implanted in defects of the knee or interverterbral disc.[228] Primary chondrocyte-embedded, chemically crosslinked ELP hydrogels containing tissue transglutaminase maintained a chondrocyte phenotype with increased sulfated glycosaminoglycan and type II collagen production as well as increased mechanical integrity in vitro.[216]

ELP fusions with the REDV or RGD sequences of the fibronectin domain for cell binding have increasingly been used in vascular graft tissue engineering to restore blood vessels.[240–242,322] ELP fusions in multiblock form consisted of Lys-incorporated, crosslinkable ELP domains to control the elastic modulus of the crosslinked scaffolds, and cell adhesion domains to attach to integrins as cell membrane proteins because fibroblasts, endothelial cells, and platelets as components of vascular tissue grafts are unable to adhere to elastin-based sequences while binding to cell-adhesion ligands.[240–242,321,322,324] Endothelial cells formed a monolayer on the chemically crosslinked ELP fusions and their growth was controlled by the frequency of cell-binding domains and the distance between the crosslinkable ELP block while Young's modulus of the ELP fusion hydrogel scaffold was close to that of native elastin in the range of 0.3 and 0.6 MPa.[321] It suggests that this class of chemically crosslinked ELP fusion hydrogel scaffolds functions as artificial ECMs and can be manipulated to control specific cellular binding and responses; furthermore, the mechanical properties of these scaffolds can be fine-tuned.

4.2.2. Three-Dimensionally Patterned Peptide Hydrogel Scaffolds

The application of original 3D patterning methodologies to control the 3D biological and biochemical properties of cell

microenvironments has led to advances in designing peptide hydrogel scaffolds. As proof of concept, Anseth and co-workers recently developed 3D patterned peptide hydrogels by sequential click reactions including copper-free [3 + 2] Huisgen cycloaddition and subsequent thiol-ene reaction by UV light.[325,326] Peptide macromolecular precursors with metalloproteinase (MMP)-cleavable sequences have dual functionalities including difluorinated cyclooctyne groups (–C≡C–) and photoreactive allyloxycarbonyl moieties (–C=C–). Hydrogelation of the peptides occurred within 5 minutes and their gel networks evolved in an hour, due to Cu-free [3 + 2] Huisgen cycloaddition between an alkyne (–C≡C–) of the peptide and an azide ($–N_3$) of a four-arm poly(ethylene glycol) (PEG) tetra-azide.[325] Subsequently, micrometer-scaled spatial patterning of the preformed peptide hydrogels was achieved by UV light-mediated thiol-ene reactions between the vinyl groups (–C=C–) of the peptides and any thiol-containing molecules. Different biological or biochemical cues to direct cell adhesion and signaling can be 3D patterned at precisely controlled spatial locations within the hydrogel scaffolds by adjusting the focal point of the laser light or two-photon irradiation in three dimensions.[3,325,326] Furthermore, the MMP cleavable sequences of the peptide hydrogels with 3D patterned reporter probes allows for detection of MMP activity and remodeling of the 3D surroundings of cells through the cleavage of cell-secreting MMPs. In addition, dynamic 3D patterns within enzyme-responsive peptide hydrogels can be achieved by spatial deposition of chemically crosslinked peptide layers composed of a series of peptide sequences containing different protease cleavage sites and spatially and temporally controlled degradation of specific 3D locations within the hydrogels.[327] This pioneering class of 3D-patterned, chemically crosslinked peptide hydrogel scaffolds allows for the creation of fine-tunable artificial ECMs as biomimetic scaffolds for tissue engineering applications.

5. Conclusions

We have reviewed a variety of biomimetic approaches to design and engineer pre-fabricated scaffolds with controlled ECM-like structures and in situ gelled hydrogel scaffolds composed of biologically inspired materials that can be used as biomimetic scaffolds for tissue engineering, regenerative medicine, and drug delivery. Although a number of goals in creating biomimetic scaffolds have been achieved, we still need to 1) gain a basic understanding of the detailed molecular mechanisms underlying the interactions between cells and the ECM; 2) determine how to fine-tune the micro- and nanoscaled structures of biomimetic scaffolds to emulate the natural ECM structures and topologies; and 3) determine how to design cell-responsive, smart, and dynamic hydrogel scaffolds that can be remodeled to mimic the bi-directional molecular interactions that occur between cells and the ECM surrounding them.[328–330] In particular, stimuli or ligand-responsive hybrid biomaterials composed of peptides and other biomolecules including nucleic acids, carbohydrates, or lipids are promising candidates for the development of "intelligent biomimetic hydrogel scaffolds". We hope that our comprehensive review of biomimetic scaffolds with advanced and sophisticated biomimetic architectures will stimulate further research and advances in the exciting fields of tissue engineering and tissue repair.

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