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Designer Self-Assembling Peptide Scaffolds for Tissue Engineering and Regenerative Medicine

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11.1 Introduction

Advancement of biology often requires development of new materials, methods, and tools that can in turn significantly accelerate scientific discoveries. The introduction of the Petri dish over 100 years ago provided an indispensable tool for culturing cells in vitro, thus permitted detailed dissection of seemingly intractable biology and physiology systems into manageable units and well-defined studies. This simple dish has had profound impact on our understanding of complex biology, especially cell biology and neurobiology.

In the manner, regenerative medicine and tissue engineering require two complementary key ingredients. One of which is the biologically compatible scaffold that can be readily adopted by the body system without harm, and the other are suitable cells including various stem cells or primary cells that effectively replace the damaged tissues without adverse consequences. However, it would be advantageous if one could apply suitable and active biological scaffolds to stimulate and promote cell differentiation, in addition to regenerating tissues without introducing foreign cells.

The field of regenerative medicine is undergoing a rapid growth, and it is impossible to cover it in a comprehensive manner in a few pages; thus in this review, we only focus on the work concerning synthetic designer self-assembling peptide scaffolds developed in our lab and our colleagues since 1992. Readers interested in advances in biomaterials can consult other articles in this book and broadly in the literature.

11.2 Scaffolds for Tissue Engineering

What are biomaterials? The definition varies. In our view, there is a distinction between
AQ1 biomaterials and biological materials. Biomaterials refer to materials that are used in medical applications in the last few decades including several synthetic polymers, poly-(D,L-lactide) (PLLA) and poly-(lactic-co-glycolic acid) (PLGA). On the other hand, biological materials refer to materials of truly biological origin including alginate, cellulose, lipid materials, chitin, and peptide- and protein-based materials, for example, collagen, silk, spider silk, and bioadhesives.

The polymer biomaterial culture systems have significantly advanced our understanding of cell–material interactions and fostered a new field of tissue engineering [1]. However, high porosity scaffolds comprising these biomaterials are often made of microfibers with a diameter of ~5–50 microns or micropores of 10–50 microns. Since the size of most cells (~5–20 μ) are similar to or smaller than these microstructures (~10–100 μ), upon attachment the cells still exhibit a 2-D topography with a curvature depending respectively on the microfiber diameters or on the pore size. In order to culture cells in a truly 3-D microenvironment, these dimensions must be significantly smaller than the cells so that the cells should be fully surrounded by the scaffolds, much like the extracellular environment.

AQ2 Polymer biomaterials are often functionalized with an RGD peptide motif or other motifs to promote desired biological activities through chemical reactions or coating. Because of their microscale sizes, their mechanical strength usually prevents further material structural adaptations from the forces exerted by the cytoskeleton of cells during their adhesion, migration, and maturation processes. Thus, although these microfibers provide an artificial extracellular environment, they are still far from the natural nanoscale extracellular matrix (ECM). In the recent years, a well-established technique, electrospinning, has been adopted to spin nanofibers of these same materials [2]; however, the harshness of the overall process and the presence of harmful chemical solvent prevent any possible addition of cells while the scaffold is forming. Attempts have been made to seed scaffold in dynamic conditions or just on their surfaces, but even if a considerable cell migration occurred, a uniform and correct seeding of cells in real 3-D matrix is still a hurdle that has to be overcome.

For the encapsulation of labile bioactive substances and living cells, physically crosslinked nanofiber scaffolds are of great interest, especially if the scaffold formation occurs under mild physiological conditions without any organic solvent processes. There is a need for process scaffolds in aqueous environment with a desired pH range, temperature, or specific catalysts that could control their microstructure properties.

11.3 Ideal Biological Scaffold

AQ3 There are a number of strategies to fabricate biological materials. However, the ideal biological scaffold should meet several requirements: (1) the building blocks should be derived from biological sources; (2) the basic units should be amenable to design and modification to achieve specific needs; (3) exhibit a controlled rate of material biodegradation; (4) exhibit no cytotoxicity; (5) promote cell-substrate interactions; (6) elicit none or little immune responses nor cause inflammation; (7) afford economically scaleable and reproducible material production, purification, and processing; (8) be readily

transportable; (9) be chemically compatible with aqueous solutions and physiological conditions; (10) and integrate in the body without harm.

11.4 Self-Assembling Peptide Scaffolds

The self-assembling peptide scaffold belongs to a class of biologically inspired materials. The first member of the family, EAK16-II (AEAEAKAKAEAEAKAK), was discovered from a segment in a yeast protein, Zuotin [3]. The scaffolds consist of alternating amino acids that contain 50% charged residues [3,4]. These peptides are characterized by their periodic repeats of alternating ionic hydrophilic and hydrophobic amino acids that spontaneously form β-sheet structures. These β-sheets have distinct polar and nonpolar surfaces. They are isobuoyant in aqueous solution and readily transportable to different environments. Upon exposure to aqueous solutions with neutral pH, ions screen the charged peptide residues and alanines (forming the nonpolar surfaces of β-sheets) of different β-sheets pack together thanks to their hydrophobic interactions in water, thus giving double-layered β-sheets nanofibers, a structure that is found in silk fibroin from silkworm and spiders. Thus, the final self-assembly step creating the peptide scaffold takes place under physiological conditions. Individual fibers are ~10 nm in diameter. On the charged sides, both positive and negative charges are packed together through intermolecular ionic interactions in a checkerboard-like manner. In general, these self-assembling peptides form stable AQ5
β-sheet structures in water, which are stable across a broad range of temperature, wide pH ranges in high concentration of denaturing agent urea and guanidium hydrochloride. The nanofiber density correlates with the concentration of peptide solution and the nanofibers retain extremely high hydration, >99% in water (5–10 mg/mL, w/v). A number of additional self-assembling peptides including RADA16-I (AcN-RADARADARADARADA-CNH2) and RADA16-II (AcN-RARADADARARADADA-CNH2), in which arginine and aspartate residues substitute lysine and glutamate, have been designed and characterized for salt-facilitated nanofiber scaffold formation.

Many self-assembling peptides that form scaffolds have been reported and the numbers are still expanding [5,6]. The formation of the scaffold and its mechanical properties are influenced by several factors, two of which are the level of hydrophobicity [7–12] and the length of peptide sequence. That is, in addition to the ionic complementary interactions, the extent of the hydrophobic residues, Ala, Val, Ile, Leu, Tyr, Phe, Trp (or single letter code, A, V, I, L, Y, P, W), and consequently the number of repeats of the self-assembling motif, can significantly influence the mechanical properties of the scaffolds and the speed of their self-assembly. The higher the content of hydrophobicity and the longer the length of the peptide sequence, the easier it is for scaffold formation and the better for their mechanical properties [7,8,11,13].

11.5 In Vitro Tissue Cultures

These new self-assembling peptide biological scaffolds have become increasingly important not only in studying spatial behaviors of cells but also in developing approaches for

a wide range of innovative medical technologies including regenerative medicine. One example is the use of the peptide scaffolds to support neurite growth and maturation [14], neural stem cell differentiation, and cardiac myocytes, bone, and cartilage cell cultures. The peptide scaffolds from RADA16-I and RADA16-II form nanofiber scaffold in physiological solutions that stimulated extensive rat neurite outgrowth, and active synapse formation on the peptide surface was successfully achieved [14].

AQ6 Navarro-Alvarez et al. [15] showed that nanofiber scaffold consists of RADA16-I can support isolated porcine hepatocytes culture and 3-D spheroidal formation for two weeks. This indicates that the hepatocytes maintained cell differentiation status. On the other hand, hepatocytes in collagen type I showed a spread shape that indicates dedifferentiation. Ammonia and drug-metabolizing capacities and albumin-producing abilities were maintained for two weeks in the hepatocytes cultured in RADA16-I scaffold, whereas there was a significant loss of those abilities in the hepatocytes cultured in collagen. This indicates that the self-assembling peptide scaffold can help maintain cell function for longer-term culture even better than naturally derived matrix in some cell types.

A method to encapsulate chondrocytes within peptide scaffolds was developed using another self-assembling peptide KLD12 (AcN-KLDLKLDLKLDL-CNH2) for cartilage repair purposes [10]. During 4 weeks of culture in vitro, chondrocytes seeded within the peptide scaffold developed a cartilage-like ECM rich in proteoglycans and type II collagen indicative of a stable chondrocyte phenotype. Time-dependent accumulation of this ECM was paralleled by increases in material stiffness indicative of deposition of mechanically functional tissue. The content of viable differentiated chondrocytes within the peptide scaffold increased at a rate that was fourfold higher than that in parallel chondrocyte-seeded agarose culture, a well-defined reference chondrocyte culture system. These results demonstrate the potential of a self-assembling peptide scaffold as a scaffold for the synthesis and accumulation of a true cartilage-like ECM in a 3-D cell culture for cartilage tissue repair. These results demonstrated peptide scaffolds are feasible for additional explorations with other tissue types.

11.6 Self-Assembling Peptide Scaffolds for Regenerative Medicine

Misawa et al. [16] showed that self-assembling peptide nanofiber scaffold developed from RADA-I promoted bone regeneration. The self-assembling peptide scaffold was injected into bone defects of mice calvaria. Saline and Matrigel were injected as controls. After 4 weeks, x-ray radiograph and histological findings showed that there were stronger bone regenerations in peptide scaffold compared to the controls. Especially, the strength of the regenerated bone was >1.7-fold higher for RADA-I peptide scaffold than for Matrigel.

Ellis-Behnke and colleagues showed that self-assembling peptide material is a promising scaffold for neural regeneration medicine [17]. In vivo application to brain wounds was carried out using postnatal day 2 Syrian hamster pups. The optic tract within the superior colliculus (SC) was completely severed with a deep knife wound, extending
AQ7 at least 1 mm below the surface. At surgery, 10 animals were treated by injection into
AQ8 the wound of 10–30 μL of 1% RADA16, 99% water, w/v). Control animals with the same brain lesion included 3 with isotonic saline injection (10 μL) and numerous additional cases, including 10 in which the dye Congo red was added into the peptide scaffold and 27 earlier animals with knife cuts and no injection surviving 6–9 days. Animals were

sacrificed at 1, 3, 6, 30, and 60 days for brain examinations. Histological specimen examinations revealed that only in the peptide-scaffold-injected animals, but not in untreated animals, the brain tissue appears to have reconnected itself together in all survival times. Additionally, axons labeled from their retinal origin with a tracer molecule were found to have grown beyond the tissue bridge, reinnervating the SC caudal to the lesion. Most importantly, functional tests proved a significant restoration of visual function in all peptide-scaffold-treated animals.

In another work published by Richard Lee's group, embryonic stem cells were suspended in RADA16-II peptide scaffold solutions and injected in the myocardium of 10-week-old mice [18]. In that study, it has been demonstrated that self-assembling peptides can be injected into the myocardium to create a 3-D microenvironment. After 7, 14, and 28 days, these microenvironments recruit both endogenous endothelial and smooth muscle cells, and exogenously injected cells survive in the microenvironments: self-assembling peptides can thus create injectable microenvironments that promote vascularization.

In addition, Lee's group also developed an appealing drug delivery strategy by using a biotinylated version of RADA-II to demonstrate a slow release of IGF-1 in infarctuated rat myocardia [19]. The biotin sandwich strategy allowed binding of IGF-1 and did not prevent self-assembly of the peptides into nanofibers within the myocardium. In conjunction with cardiomyocytes transplantation, the strategy showed that cell therapy with IGF-1 delivery by biotinylated nanofibers significantly improved systolic function after experimental myocardial infarction.

Remarkably, since the building blocks of this class of designer peptide scaffolds are made of pure natural L-amino acids, RADA16, unlike most of the other synthetic microfibers, has been shown not to elicit detectable immune response nor inflammatory reactions in animals [16–19], and the degraded natural amino acid products can be reused by the body. Therefore, this class of scaffold may be useful as a bioreabsorbable scaffold for tissue repair and neuroengineering to alleviate and treat a number of trauma and neurodegeneration diseases as well as other tissue injuries, damage, and aging.

Alternative strategies are under evaluation to address the directionality and alignment of these scaffolds; for example, the nanofibrous scaffolds do not have a predetermined porosity, pore orientation, and a predetermined 3-D oriented architecture, by using microfluidic or magnetic approaches to drive the self-assembling process.

11.7 Designer Self-Assembling Peptide Scaffolds

Although self-assembling peptides are promising scaffolds, they show no specific cell interaction because their sequences are not naturally found in living systems. The next logical step is to search active and functional peptide motifs from a wealth of cell biology literature; thus, the 2nd generation of designer scaffolds will significantly enhance their interactions with cells and tissues [20–22].

The simplest method to incorporate the functional motifs is to directly synthesize it by extending the motifs onto the self-assembling peptides themselves (Figure 11.1) [22,23]. The functional motifs are on the C-termini since peptide synthesis starts from C-termini to avoid deletion of the functional motifs during synthesis. Usually, a spacer comprising two glycine residues is added to guarantee a flexible and correct exposure of the motifs to cell surface receptors. Different functional motifs in various ratios can be incorporated

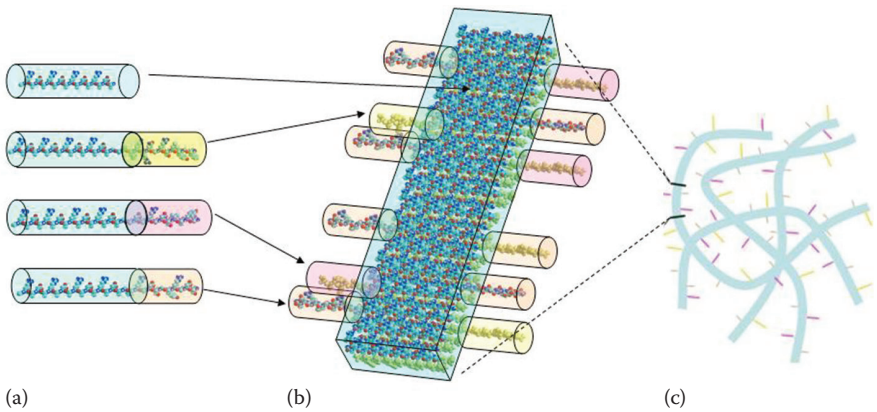

FIGURE 11.1
Schematic illustration of the designer self-assembling peptide scaffold. (a) Direct extension of the self-assembling peptide sequence by adding different functional motifs. Light turquoise cylinders represent the self-assembling backbone and the yellow, pink, and tan lines represent various functional peptide motifs. (b) Molecular model of a self-assembling peptide nanofiber with functional motifs flagging from both sides of the double ß-sheet nanofibers. Either mono or multiple functional (or labeled) peptide motifs can be mixed at the same time. The density of these motifs can be easily adjusted by simply mixing them in various ratios, 1:1–1,000,000, or more before the assembling step. (c) They then will be part of the self-assembled scaffold.

in the same scaffold. Upon exposure to the solution with neutral pH, the functionalized sequences self-assemble leaving the added motifs flagging on both sides of each nanofiber. Nanofibers take part to the overall scaffold thus giving microenvironments functionalized with specific biological stimuli (Figure 11.1).

AQ9 The incorporation of functionalized peptide into the nanofiber was indicated by AFM [23]. 1% (w/v) peptide solution of RADA16-I and the functionalized peptide of RADA16-I AQ10 with 2-unit RGD binding sequence PRG (PRGDSGYRGDS) were examined. The mixed peptide solution of RADA16-I and the PRG functionalized peptide at a ratio of 1:1 was also examined using AFM tapping mode. Figure 11.2 shows AFM images of the peptide solutions. The nanofibers in aqueous solutions were observed in RADA16-I and all RADA16-I mixed solutions, although no fiber formation was observed in solely PRG functionalized peptide solution. These results were confirmed by visual inspection of increasing viscosity of the peptide scaffold solutions. We observed an increase in the fiber thickness in the mixed solution (29.5 ± 3.1 nm) from RADA16-I solution (16.3 ± 1.4 nm). The width of the peptide fiber thickness was correlated with the model shown in Figure 11.1. These results imply that PRG functionalized peptides were integrated in nanofiber when it is mixed.

The self-assembling peptide scaffolds with functional motifs can be commercially produced with a reasonable cost. Thus, this method can be readily adopted for widespread uses including the study on how cell interacts with their local and microenvironments, cell migrations in 3-D, tumor and cancer cell interactions with the normal cells, cell process and neurite extensions, cell-based drug test assays, and other diverse applications.

We have produced different designer peptides from a variety of functional motifs with different lengths [22,23]. We showed that the addition of motifs (up to 12 additional residues) to the self-assembling peptide RADA16-I did not inhibit self-assembling properties and nanofiber formations. Although their nanofiber structures appear to be indistinguishable from the RADA16-I scaffold (Figure 11.3), the appended functional motifs significantly influenced cell behaviors [22–25].

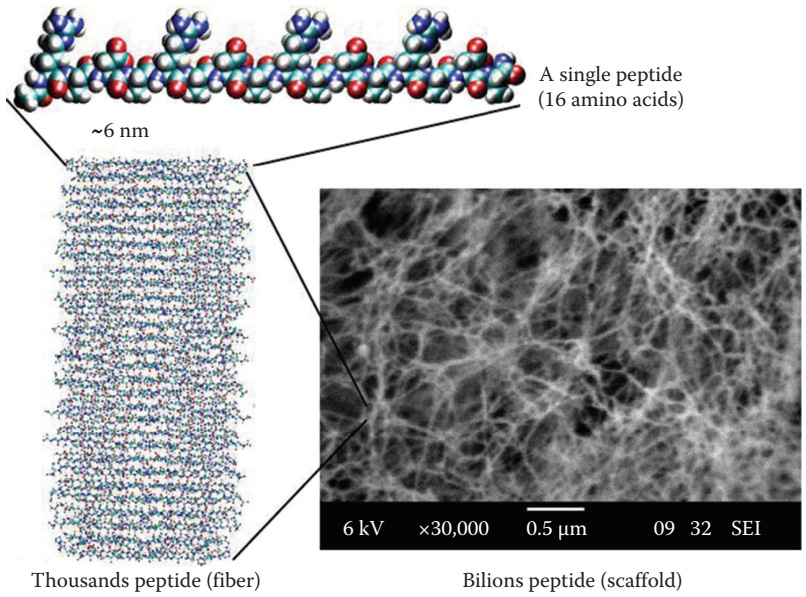

FIGURE 11.2
The designer self-assembling peptide nanofiber scaffold. A single peptide, ~6 nm, is shown. Thousands of peptides self-assemble to form a single nanofiber; trillions of peptides or billions of nanofibers form the scaffold, which contain ~99.5% water and 0.5% peptide materials. Positive and negative charges are labeled in blue and in red colors, respectively.

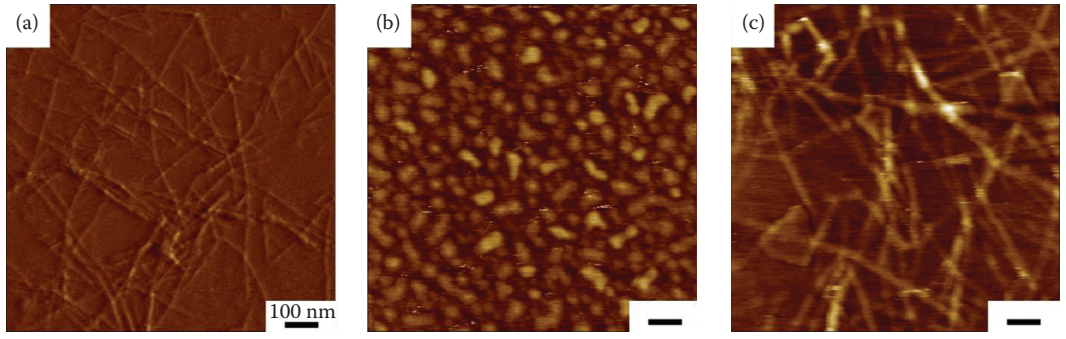

FIGURE 11.3
Tapping mode AFM images of 1% w/t peptides solution of (a) RADA16-I, (b) PRG functionalized peptide, and (c) PRG functionalized peptide + RADA16-I (1:1). The bar represents 100 nm. The nanofiber formation is seen in (a) RADA16-I and (c) PRG + RADA16-I (1:1). An increase in the fiber thickness in (c) PRG + RADA16-I (1:1) (29.5 ± 3.1 nm) from (a) RADA16-I (16.3 ± 1.4 nm) was observed, which correlated to the width of the peptide fiber modeled in Figure 11.1.

Using the designer self-assembling peptide nanofiber system, every ingredient of the scaffold can be defined and combined with various functionalities including the soluble factors. This is in sharp contrast with a 2-D Petri dish where cells attach and spread only on the surface; cells reside in a 3-D environment where the ECM receptors on the cell membranes can bind to the functional ligands appended to the peptide scaffolds (Figure 11.4). It is possible that higher tissue architectures with multiple cell types, rather than

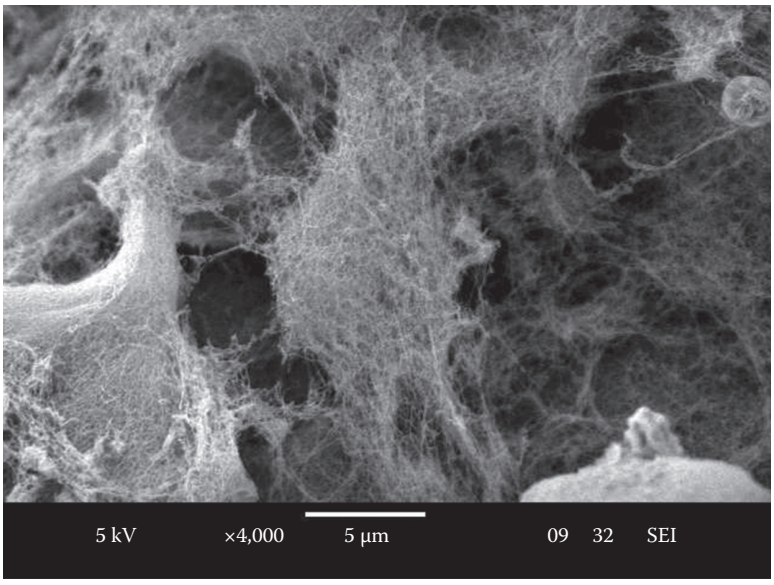

FIGURE 11.4
SEM image of a cell embedded in a 3-D designer self-assembling peptide nanofiber scaffold. The individual nanofiber scaffold completely wraps cell body and membrane thus bringing the chosen functional motifs all over the cell membrane.

monolayers, could be constructed using these designer 3-D self-assembling peptide nanofiber scaffolds.

Even if only a fraction of functionalized motifs on the 3-D scaffold are available for cell receptor binding, cells likely receive more external stimuli than when in contact with coated 2-D Petri dishes or RGD- or other motif-coated polymer fibers in micron scale, which is substantially larger than the cell surface receptors and in most cases, larger than the cell themselves. These cells are not in real 3-D; rather, they are in 2-D wrapping around the polymers with a curvature depending on the diameter of the polymers. In a 2-D environment, where only one side of the cell body is in direct contact with the surface, receptor clustering at the attachment site may be induced; on the other hand, the receptors for growth factors, cytokines, nutrients, and signals are on the other side that expose directly with the culture media. Thus, cells may become partially polarized. In the 3-D environment, the functional motifs on the nanofiber scaffold surround the whole cell body in all dimensions and the factors may form a gradient in 3-D nanoporous microenvironment.

11.7.1 Designer Peptide Scaffolds for Cell differentiation and Migration

The designer self-assembling peptide nanofiber scaffolds have been shown to be an excellent biological material for 3-D cell cultures and capable to stimulate cell migration into the scaffold as well as for repairing tissue defects in animals by adding specific cell binding and/or chemotactic sequences. We developed several peptide nanofiber scaffolds designed specifically for osteoblasts [23]. We designed one of the pure self-assembling peptide scaffolds RADA16-I through direct coupling to short biologically active motifs. The motifs included osteogenic growth peptide ALK (ALKRQGRTLYGF) bone-cell secreted-signal peptide, osteopontin cell adhesion motif DGR (DGRGDSVAYG), and 2-unit RGD binding

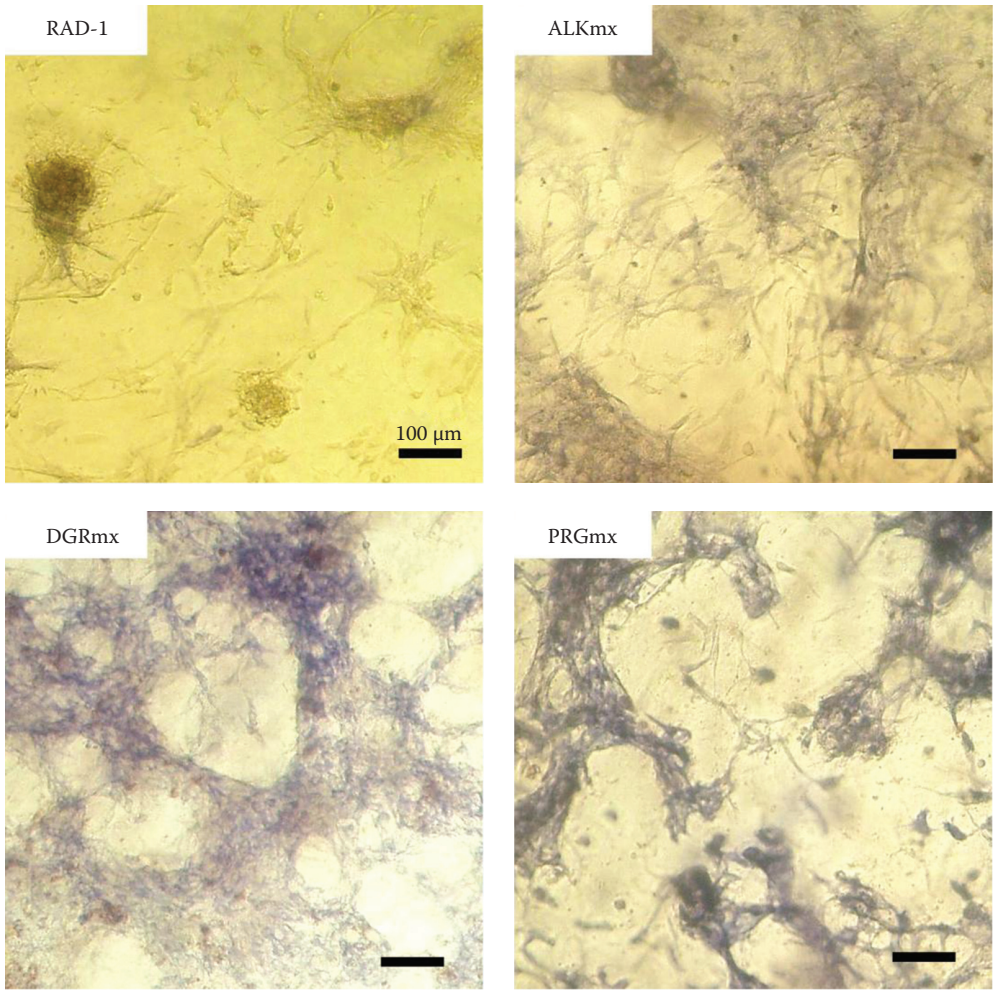

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FIGURE 11.5
ALP staining images after culturing on the different hydrogels for 2 weeks. The bar represents 100 μm. RAD-I–RADA16-I 1% (w/v), ALKmx–ALK functionalized peptide 1% (w/v) + RADA16-I, DGRmx–DGR functionalized peptide 1% (w/v) + RAD, PRGmx–PRG functionalized peptide 1% (w/v) + RADA16-I (all mixture ratio is 1:1). The darkness correlates with the high ALP activity. RADA16-I shows low cell adhesion to the hydrogel and the cells are aggregated. The cell attachment increases of DGR and PRG were considered to be caused by RGD cell attachment sequence. ALK, DGR, and PRG showed higher ALP activities compared to RADA16-I, especially staining intensity of PRG.

sequence PRG (PRGDSGYRGDS). We made the new peptide scaffolds by mixing the pure RAD16 and designer peptide solutions. Compared to pure RAD16 scaffold, we found that these designer peptide scaffolds significantly promoted mouse preosteoblast MC3T3-E1 cell proliferation. Moreover, alkaline phosphatase (ALP) activity and osteocalcin secretion, which are early and late markers for osteoblastic differentiation, were also significantly increased. The maker results were confirmed by ALP staining (Figure 11.5). We demonstrated that the designer, self-assembling peptide scaffolds promoted the proliferation and osteogenic differentiation of MC3T3-E1. Under the identical culture medium condition, confocal images unequivocally demonstrated that the designer PRG peptide

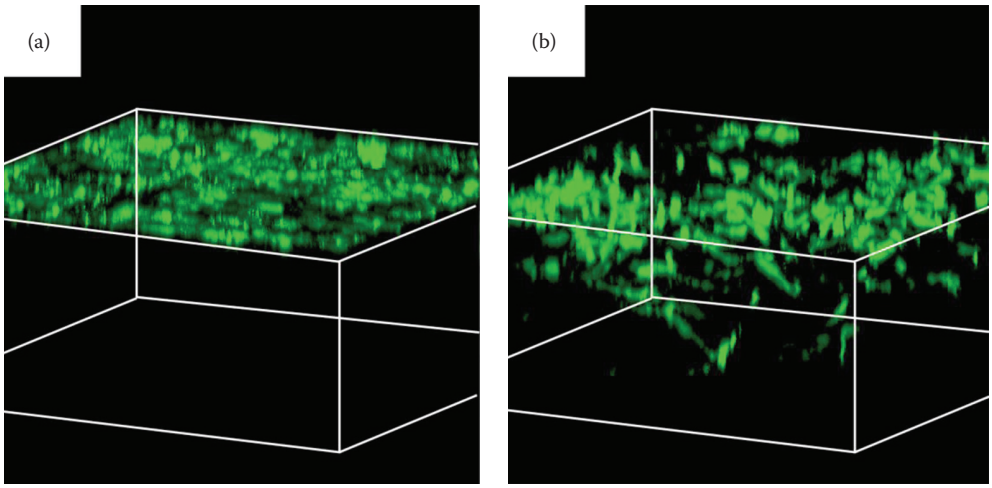

FIGURE 11.6
Reconstructed image of 3-D confocal microscope image of culturing on the different scaffolds consisting of different mix ratio of RADA16 1% (w/v) and PRG 1% (w/v) using calcein-AM staining. The bar represents 100 μm. (a) PRG 10% and (b) PRG 70%. In the case of 10% PRG scaffold, the cells were attached on the surface of the scaffold, whereas the cells were migrated into the scaffold in the case of 70% PRG scaffold. There is a drastic cell migration into the scaffold with higher concentration of PRG motif.

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scaffold stimulated cell migration into the 3-D scaffold (Figure 11.6) [23]. Our results suggest that these designer peptide scaffolds may be very useful for promoting bone tissue regeneration.

In addition to laminin-derived self-assembling peptides previously studied by adding a long alkyl chain that promotes self-assembly [24], another study evaluates common fibronectin- and collagen-derived sequences as well [25].

11.8 Why Designer Self-Assembling Peptide Scaffolds?

Why one should choose designer self-assembling peptide scaffolds while there are a large number of biomaterials on the market and some have already been approved by the Food and Drug Administration (FDA)? The advantage of using the designer peptide nanofiber scaffolds is severalfold. (1) One can readily modify the designer peptides at the single amino acid level at will, inexpensively and quickly. This level of modification is impossible with Matrigel and other polymer scaffolds. (2) Unlike Matrigel, which contains unknown ingredients and quality that varies from batch to batch, the designer self-assembling peptide scaffolds belong to a class of synthetic biological scaffolds that contain pure components and every ingredient is completely defined. (3) Because these designer peptide scaffolds are pure with known motifs, it can be used to study controlled gene expression or cell signaling process. Thus, these new designer nanofiber scaffolds proved to be promising tools to study cell signal pathways in a selective way not possible with any substrates including Matrigel and collagen gels that result in confusing cell signaling activation. (4) The initiation of the self-assembly process is through change of ionic strength at

the physiological conditions without temperature influence. This is again unlike collagen gels, for which the gelation is through change of temperature that can sometimes induce unknown biological process including cold or heat shocks. (5) These scaffolds provide the opportunity to incorporate a number of different functional motifs and their combinations to study cell behavior in a well-defined ECM-analog microenvironment, not only without any chemical cross-link reactions but also fully bioreabsorbable scaffolds.

In the development of new biological materials, particularly those biologically inspired AQ13 nanoscale scaffolds mimicking in vivo environment that serve as permissive substrates for cell growth, differentiation and biological function is the most actively pursuit area, which in turn could significantly advance regenerative medicine. These materials will be useful not only for furthering our understanding of cell biology in 3-D environment but also for advancing medical technology, tissue engineering, regenerative biology, and medicine.

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AUTHOR QUERIES

[AQ1] Please check if edit to the sentence starting "Biomaterials refer..." is okay.
[AQ2] Please provide the expansion of the acronym "RGD," if appropriate.
[AQ3] Please check if edit to the sentence starting "However, the ideal..." is okay.
[AQ4] Please check if the fixed running head is okay.
[AQ5] Please check the sentence starting "In general, these..." for clarity.
[AQ6] Please check if edit to the sentence starting "Navarro-Alvarez et al...." is okay.
[AQ7] Please provide opening parenthesis in sentence starting "At surgery...."
[AQ8] Please check if edit to the sentence starting "Control animals with..." is okay.
[AQ9] Please provide the expansion of the acronym of "AFM," if appropriate.
[AQ10] Please provide the expansion of the acronym "PRG," if appropriate.
[AQ11] Please provide the part labels for figure 11.5 and also insert this in the captions.
[AQ12] Please provide the expansion of the acronym "AM," if appropriate.
[AQ13] Please check if edit to the sentence starting "In the development..." is okay.
[AQ14] We have renumbered the reference list to maintain sequential order. Please confirm the appropriate citation to Refs. [23–26].