CHAPTER

4

Designer Self-Assembling Peptide Scaffolds for 3D Tissue Cell Cultures

Fabrizio Gelain, Xiumei Wang, Akihiro Horii, Angus Hucknall, Sotirios Koutsopoulos, and Shuguang Zhang

Center for Biomedical Engineering NE47-379, Center for Bits & Atoms, Massachusetts Institute of Technology, Cambridge, MA, Corresponding author: Shuguang Zhang, address: Center for Biomedical Engineering NE47-379, Center for Bits & Atoms, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, United States, phone: 1-617-258-7514, fax: 1-617-258-5239, e-mail: Shuguang@mit.edu

Abstract

Biomedical researchers have become increasingly aware of the limitations of conventional 2D tissue cell cultures. They are now testing 3D cell culture systems, something between a Petri dish and a mouse. The important implications of 3D tissue cell cultures for basic cell biology, tumor biology, high-content drug screening, regenerative medicine, and beyond are far-reaching. It has become more apparent that 3D cell cultures offer a more realistic microenvironment for the cells in the nanofiber scaffolds where the cells can be observed and manipulated. A class of designer self-assembling peptide nanofiber scaffolds provides an ideal 3D culture system. The time has come to address the 3D questions because quantitative biology requires in vitro culture systems that more authentically represent the cellular microenvironment in a living organism. In doing so, in vitro experimentation can become truly more predictive of in vivo systems. This chapter describes methods to work on designer self-assembling peptide nanofiber scaffolds for 3D tissue cell cultures.

Key terms

3D cell culture
designer self-assembling peptides
hydrogel
matrix
nanofibers
scaffold
unidirectional cell migration

4.1 Introduction

Advancement of biology often requires development of new materials, methods, and tools. 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 a profound impact on our understanding of complex biology, especially cell biology and neurobiology. However, the Petri dish culture system, including multiwell plates, glass cover slips, and so fourth, is less than ideal for several reasons:

- It is a two-dimensional (2D) system, which is in sharp contrast to the 3D environment of natural tissues in animals and plants.
- The Petri dish surface without a coating is rigid and inert, again in sharp contrast to the in vivo environment where cells intimately interact with the extracellular matrix and with each other.
- The tissue cell monolayers cultured on coated 2D surface, such as poly-L-lysine, collagen gels, fibronectin, laminin, and Matrigel as well as other synthetic materials containing segments of adhesion motifs, have only part of the cell surface attached to the materials and interact neighboring cells. The remaining parts are often directly exposed to the culture media, unlike the tissue environment where every cell intimately interacts with neighboring cells and/or the extracellular matrix.
- Biomolecular diffusion in 2D cultures is drastically different from those in 3D culture systems. In 2D culture systems, cytokines, chemokines, and growth factors quickly diffuse in the media across the culture dish. This is again in sharp contrast to the in vivo environment where chemical and biological gradient diffusion systems play a vital role in signal transduction, cell-cell communications, and development.
- Cells cultured on a 2D Petri dish are not readily transportable; that is, it is nearly impossible to move cells from one environment to another without incurring changes in the cell-material and cell-cell interactions. For example, cell collections using trypsinization or mechanical scrap using rubber policeman have adverse effects on cell-environment interactions. In contrast, cells cultured on 3D substrates are more readily transportable without significantly harming cell-material and cell-cell interactions, thus providing a realistic way to study cell biology.

We believe that the development of new biological materials, particularly those biologically inspired nanoscale scaffolds mimicking the in vivo environment and serve as permissive substrates for cell growth, differentiation, and biological function is key. These materials will be useful not only in furthering our understanding of cell biology, but also for advancing biotechnology, tissue engineering, regenerative biology, and medicine.

The ideal biological scaffold should meet several criteria.: The building blocks should be derived from biological sources; basic units should be amenable to design and modification to achieve specific needs; exhibit a controlled rate of material biodegradation; exhibit no cytotoxicity; promote cell-substrate interactions, elicit no or little immune responses and inflammation; afford economically scaleable material production, purification and processing; be readily transportable; be chemically compatible with aqueous solutions and physiological conditions; and integrate with other body tissues.

4.1.1 Discovery and development of self-assembling peptide scaffolds

Shuguang Zhang serendipitously discovered the self-assembling peptides while sequencing a yeast protein *Zuotin* in 1990. It took him more than a year to understand how the seemingly soluble short peptides underwent self-assembly to form naked-eye visible materials. He and his colleagues filed a U.S. patent application in 1992 (issued in 1997) and published the yeast protein [1] where the first self-assembling peptide was identified.

We therefore have extensive experience in this class of biological materials made from the designer self-assembling peptides [2–4]. These self-assembling peptides consist of greater than 99% water (w/v). They form a hydrogel when exposed to physiological media or salt solution [2–7]. The constituents of the scaffold are amphiphilic peptides that have alternating repeating units of positively charged lysine or arginine and negatively charged aspartic acid and glutamic acid. The unmodified scaffolds consist of alternating amino acids that contain 50% charged residues [2–4]. These peptides are characterized by their periodic repeats of alternating ionic hydrophilic and hydrophobic amino acids. Thus, they have tendency to form beta sheets that have distinct polar and nonpolar sides [2–4].

The first self-assembling peptide, EAK16-II, a 16 amino acid peptide, was found in a segment in a yeast protein, zuotin, which was originally characterized by binding to left-handed Z-DNA. A number of additional self-assembling peptides including RAD16-I and RAD16-II, in which arginine and aspartic acid residues substitute lysine and glutamic acids have been designed and characterized for salt-facilitated scaffold formation [2–7]. Stable macroscopic matrix structures have been self-organized through the self-assembly of aqueous peptide solutions introduced into physiological salt-containing solutions. Several peptide scaffolds have been shown to support cell attachment of a variety of mammalian primary and tissue culture cells [2–7].

4.1.2 The nanofiber structure of the peptide scaffold

The peptide scaffolds consist of individual interwoven nanofibers. The individual fibers are approximately ~10–20 nanometers in diameter. The fiber density correlates with the concentration of peptide solution that is used to produce the materials. Generic observation with scanning electron microscope revealed a nanofiber network and pore (diameters ranging from 50 to 200 nm) ensemble similar to those found with biologically derived substrates like Matrigel [5]. The mechanical properties of the scaffold and their self-assembly process have also been investigated. Rheology was used as a method for comparison of the relative mechanical stiffness of the assembled peptides. The storage moduli (G′) were measured at low frequencies. This information is useful in describing the type of mechanical environment that seeded cells are presented with and can serve as a macroscopic measure of the extent of interactions formed between assembled peptides The 1% w/v self-assembling peptides like RADA16 (G′ = 1630Pa), were generally found to exhibit reduced storage moduli when compared to Matrigel (G′ = 5408 Pa). Upon increasing the peptide concentration, the storage moduli can be significantly augmented to meet the requirements of the specific research application (cartilage regeneration, stem cell differentiation, and so forth).

4.1.3 A generic biological scaffold

The self-assembling peptide scaffold belongs to a class of biologically inspired materials. The self-assembly event creating the peptide scaffold takes place under physiological conditions. They float in aqueous solution and are readily transportable to different environments. Nanostructures formed by RADA16-I self-assembling peptide are depicted in Figure 4.1; a molecular interpretation of reassembly of RADA16-I is described in Figure 4.2. These new biological materials have become increasingly important not only in studying 3D spatial behaviors of cells, but also in developing approaches for a wide range of innovative medical technologies. One example is in the use of the peptide scaffolds to support neurite growth and differentiation. We reported that peptide scaffolds from RADA16-I and RAD16-II are a permissible substrate for primary neurons isolated from the cerebellum and hippocampus of newborn rats and mice to form extensive neurite outgrowth and active synapses on the peptide surface [3].

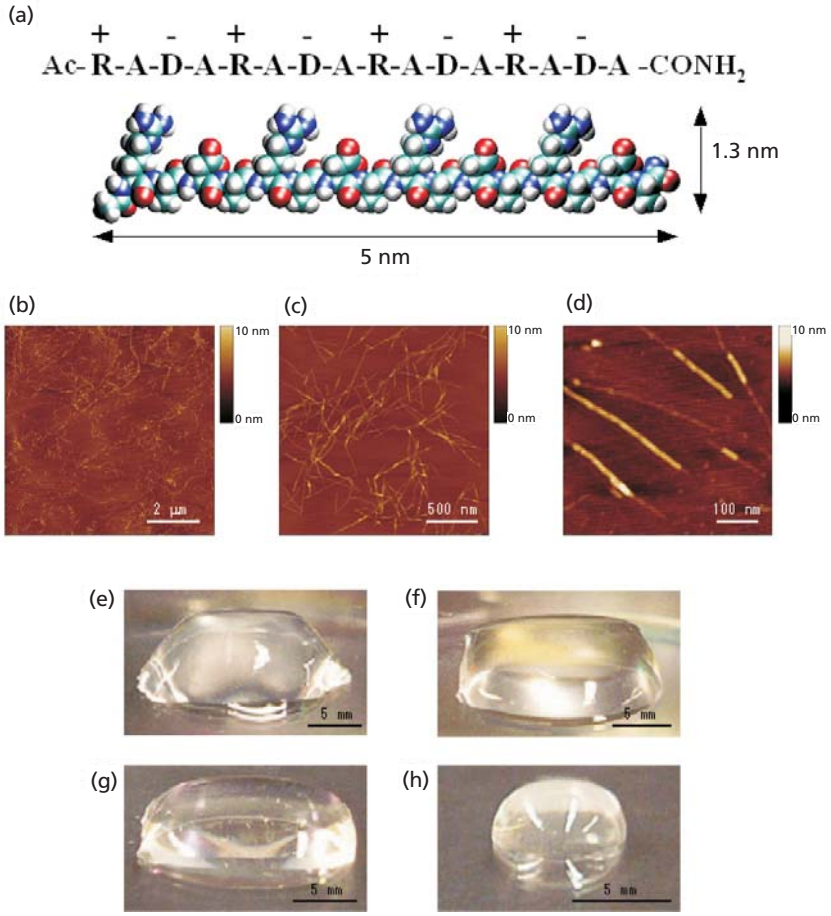

Figure 4.1 Peptide RADA16-I. (a) Amino acid sequence and molecular model of RADA16-I—the dimensions are ~5-nm long, 1.3-nm wide, and 0.8-nm thick; (b) AFM images of RADA16-I nanofiber scaffold, 8 μm × 8 μm; (c) 2 μm × 2 μm; (d) 0.5 μm × 0.5 μm. Note the different height of the nanofiber, ~2–5 nm, in (d) suggesting either a single or a double layer structure. Photographs of RADA16-I hydrogel at various conditions: (e) 0.5 wt% (pH 7.5); (f) 0.1 wt% (pH 7.5); and (g) 0.1 wt% (pH 7.5, PBS) before sonication. (h) Reassembled RADA16-I hydrogel after 4 time sonications, respectively. (Image courtesy of Hidenori Yokoi.)

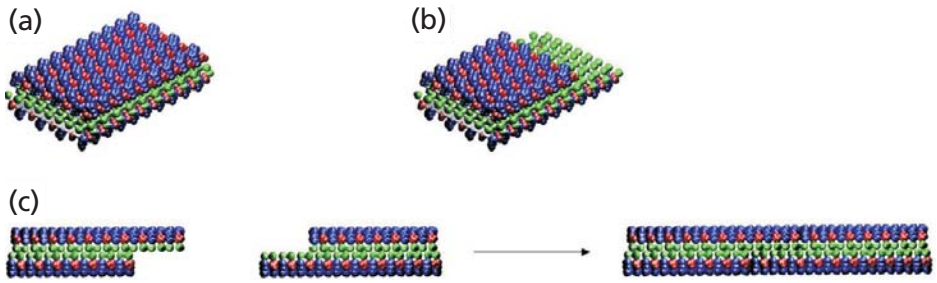

Figure 4.2 A proposed reassembly molecular model of self-assembling RADA16-I peptides. When the peptides form stable β-sheets in water, they form intermolecular hydrogen bonds along the peptide backbones. The β-sheets have two distinctive sides, one hydrophobic with an array of alanines and the other with negatively charged aspartic acids and positively charged arginines see Figure 6.2(a)]. These peptides form antiparallel β-sheet structures. The alanines form overlap packed hydrophobic interactions in water, a structure that is found in silk fibroin from silkworm and spiders. On the charged sides, both positive and negative charges are packed together through intermolecular ionic interactions in a checkerboard-like manner. These nanofiber fragments can form various assemblies similar to restriction-digested DNA fragments with (a) blunt ends and (b) semiprotruding ends. (c) These fragments with protruding and blunt ends could reassemble readily through hydrophobic interactions and hydrogen bonds. (Image courtesy of Hidenori Yokoi.)

4.1.4 Peptide scaffold fosters chondrocyte extracellular matrix production

In choosing a scaffold for cartilage repair, it is important to identify a material that can maintain high rates of proliferation of chondrocytes and high rates of chondrocyte synthesis of specific ECM macromolecules including type II collagen and glycosaminoglycans (GAGs) until they evolve into steady state tissue maintenance. We first used a RADA16 scaffold but it did not give the optimal results because it is a rather weak hydrogel. We then designed the KLD12 (n-KLDLKLDLKLDL-c) peptide scaffold, reasoning that since leucine is more hydrophobic than alanine, the leucines would likely pack more tightly in the nanofibers in aqueous conditions and thus provide a higher mechanical strength of the scaffold [4].

We used the self-assembling peptide KLD12 scaffold for cartilage repair and developed a method to encapsulate chondrocytes within the peptide scaffold. 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 the deposition of mechanically functional tissue. The content of viable differentiated chondrocytes within the peptide scaffold increased at a rate that was 4-fold higher than that in parallel chondrocyte-seeded agarose culture, a reference chondrocytes culture system. These results demonstrate the potential of self-assembling peptide scaffolds for the synthesis and accumulation of a true cartilage-like ECM in a 3D cell culture for cartilage tissue repair. The peptide KLD12 used in this study represents a designed self-assembling peptide made through molecular engineering that can be modified to suit specific cell and tissue application interests [4].

These results suggest that different cell types have different requirements for the scaffolds. We therefore systematically tailor-make peptide scaffolds for a variety of cell types.

4.1.5 Designer peptides appended with active motifs

A number of functional peptide motifs have been identified from extracellular matrix proteins. Some of the most important motifs are from collagens and laminins. Others comprise tissue-specific proteins including osteopontin, osteocalcin, osteonectin, nidogen, and netrins.

These functional motifs have been directly coupled on to the self-assembling peptide RADA16 and other self-assembling peptides, so as to obtain functional peptides linked to the C-termini of self-assembling cores. This method can directly introduce biological functions into the peptide scaffolds. After self-assembly of the nanofibers, these motifs are part of the local microenvironment. Many bioactive motifs have been selected to create a microenvironment niche to foster specific cell cultures. Since these functional motifs are modular, we can tailor-make and mix them in a combinatorial manner to achieve the optimal benefit or recreate an in vivo–like microenvironment.

It must be emphasized that the peptide nanofiber 3D scaffolds only need to create a temporary or initial scaffolding microenvironment to foster cell attachment, proliferation, migration, or differentiation. Cells may produce their own extracellular matrix proteins during that initial period. These peptide scaffolds will then be naturally degraded and the degradation product, amino acids, can be reused by cells.

Thus we developed a novel class of designer self-assembling peptides appended with a broad set of biological active motifs. A schematic representation is depicted in Figure 4.3. Blue lines represent generic self-assembling peptides (sapeptide); red, green, purple, yellow, and brown lines represent various functional peptide motifs. The functional motifs are on the C-termini since peptide synthesis starts from the C-termini to avoid imperfect coupling deletion during synthesis (~99% coupling per step). Self-assembling peptides sharing the same self-assembling backbone and showing different functional motifs can also be mixed together within the same scaffolds (Figure 4.4). It is generally known that when longer peptides are made, deletion may occur due to the coupling efficiency of the synthetic peptide chemistry. In order to preserve the fidelity of the func-

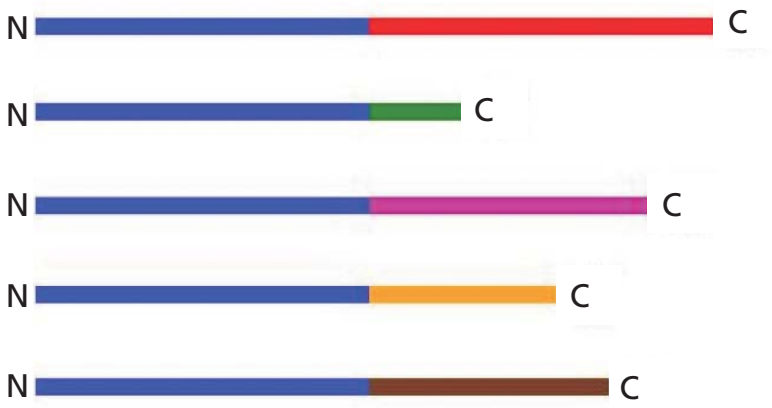

Figure 4.3 Direct extension of the functional motifs from the self-assembling peptides. The blue lines represent the sapeptide; the red, green purple, yellow, and brown lines represent various functional peptide motifs. The functional motifs are on the C-termini since peptide synthesis starts from C-termini to avoid functional motif deletion during synthesis. Since the self-assembling core has repeated motifs, any one amino acid deletion does not significantly alter its self-assembly.

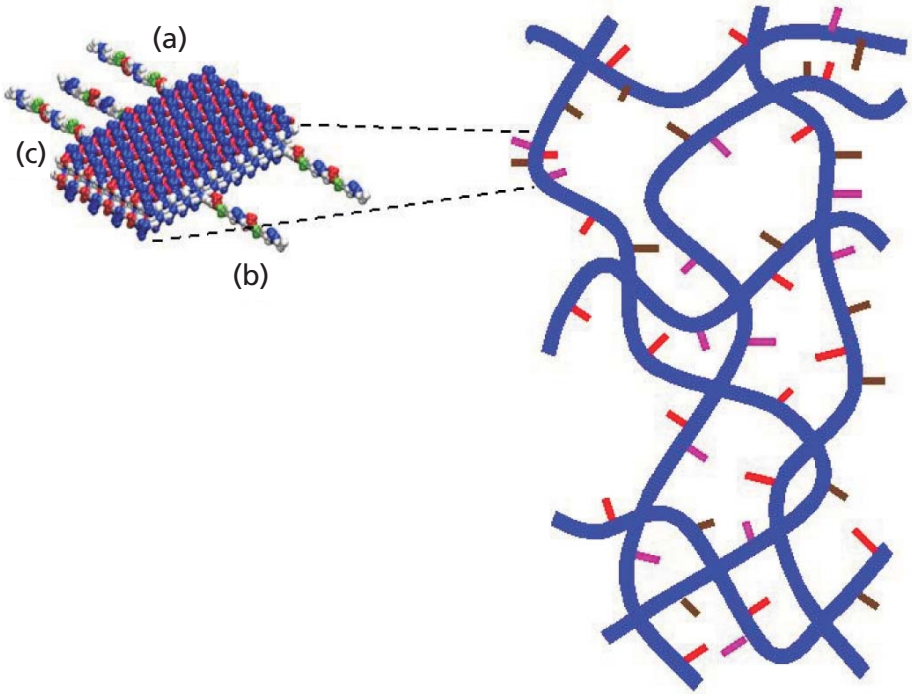

Figure 4.4 Schematic representation of a self-assembling peptide scaffold modified with various functional motifs. The density of these motifs can be easily adjusted simply by mixing different functionalized peptides in various ratios, 1:1, 1:2, 1:4, 1:10, 1:100, 1:1,000 or more depending on cell responsiveness. Either mono or multiple functional peptide motifs, labeled as A, B, and C, can be mixed at the same time.

tional peptide, they are designed to be on the C-termini in order to prevent residue alterations during chemically solid phase peptide synthesis, which has ~98% for coupling efficiency. The sequence of the sapeptide has repeating units; one deletion does not significantly alter its ability to undergo self-assembly.

A spacer comprising of two glycines is usually placed between the self-assembling sequence and the functional motif in order to provide a "joint" with sufficient degrees of freedom necessary for a correct exposure of the functional motifs to cell membrane receptors.

4.2 Materials

The RAD16 peptide scaffold solution (1% w/v) was purchased as PuraMatrix from BD Bioscience (Bedford, Massachusetts). The designer peptides with active motifs were custom-synthesized (CPC Scientific, San Jose, California). Alternatively, the functionalized designer peptides can also be in-house synthesized with a CEM Liberty Microwave Peptide Synthesizer (Matthews, NC). The peptides were dissolved in water at a final concentration of 1% (w/v) and sonicated for 20 minutes (Aquasonic, model 50T, VWR, New Jersey). Peptides can be easily produced via solid phase F-moc synthesis and purified via standard HPLC techniques.

4.3 Reagents

Standard reagents are commercially available. To maintain the tissue cell lines, penicillin/ streptomycin, the minimal essential medium, and FBS are available from Invitrogen Corp. (Carlsbad, California). Ascorbic acid and β-glycerophosphate, and the Alkaline Phosphatase Fluorescence Assay Kit 85L-2 histological assay is available from Sigma Chemical Co. (St. Louis, Missouri). The PicoGreen dsDNA Quantification Kit (P-7589) and Live/Dead Viability Kit (L-3224) are from Invitrogen (Eugene, Oregon). The Mouse Osteocalcin EIA Kit for Osteocalcin and Calcein-AM Staining is from Biomedical Technologies, Inc. (Massachusetts). Matrigel GF reduced from EHS sarcoma is available from BD Biosciences.

4.4 Methods

4.4.1 Peptide solution preparation

Double lyophilized designer peptides are custom-synthesized by CPC Scientific (San Jose, California).

1. These peptides are dissolved in MilliQ water at a final concentration of 1% w/v (10 mg/ml).
2. They are then sonicated for 30 minutes (Aquasonic, model 50T, VWR, New Jersey).
3. After sonication, they are filter-sterilized (Acrodisc Syringe Filter, 0.22-μm HT Tuffrun Membrane, Pall Corp., Ann Arbor, Michigan) for future use.
4. The designer functionalized peptide solutions (PFS and/or SKP) are mixed in a volume ratio of 1:1, 1:2, 1:4, 1:100 with 1% pure RADA16-I solution to obtain 1% functionalized peptide mixtures.

4.4.2 Designer peptide synthesis and scaffold preparation

Cell viability and differentiation assay tests are carried out.

1. Add 30 μl per well of a 96-well plate of a 1% (w/v) peptide in sterile water solution aqueous solution of either generic or functionalized peptide hydrogel so as to evenly cover the bottom surface of each well, resulting in ~30-μm gel layer thickness (BD Biosciences, Bedford, Massachusetts).
2. Very slowly add 200 μl per each well of basal medium along the side of well.
3. The mix ratio between RADA16 and PRG varied from 100:0 (PRG 0%), 99:1 (1% PRG), 90:10 (10% PRG), 60:40 (40% PRG), 30:70 (70% PRG), up to 0:100 (100% PRG).
4. Allow the peptides to self-assemble at 37°C for 30 minutes and rinse once with medium to wash away any residual acid remaining from peptide synthesis and purification.
5. In the case of SEM imaging both for Matrigel and the peptide hydrogels, the total amount of hydrogel scaffold is reduced to 10 μl, and higher concentrations of peptides (2% w/v or 3% w/v) are preferred to guarantee the necessary scaffold stiffness.

4.4.3 Culture cells in plate inserts

Each of the peptide solutions is directly loaded in the tissue culture plate inserts (10-mm diameter, Millicell-CM, Millipore, Massachusetts). The culture medium is added to induce hydrogel formation.

1. Cells are suspended in 10% sucrose just before seeding. Quickly mix 20 μl of cell suspension with 100 μl of peptide solution and then add the mixture into the insert. Very carefully layer the 400-μl medium onto the gel for gelation. Incubate 10 minutes at 37°C and then change medium for another 30 minutes of incubation. Change two more times to equilibrate the gel to physiological pH for 3D cell culture.
2. When culture cells are on the inserts, the peptide scaffolds in the inserts are washed twice using PBS to remove any residual acid remaining from peptide synthesis and purification and rise to pH 7.4.
3. For cell staining and visualization, a 4-μM calcein AM solution is added to the insert and incubated for 1 hour in the incubator at 37°C.
4. The inserts are rinsed well by PBS twice and the cells are examined via a fluorescence-inverted microscope.

4.4.4 Cell culture system

1. The scaffolds 1% (w/v) are prepared as pure RADA16 or mixed with others at a ratio of 1:1 (v/v) (RADA16: ALK, DGR, or PRG).
2. Each solution is sonicated for 30 minutes, loaded (100 μl) on top of a cell culture insert (10-mm diameter, Millicell-CM, Millipore, Massachusetts), and allowed to form a layer ~3 mm thick.
3. The maintenance medium described later is gently added on the top of the scaffold to induce gelation.
4. The system is incubated at 37°C for an hour.
5. Then the medium inside the insert is exchanged by the maintenance medium and the outside of the insert is filled with the maintenance medium and incubated for half a day within a cell culture incubator at 37°C.
6. Cells are plated at 2×10^4 cells on the hydrogels in the inserts. The cells are cultured in the maintenance medium day 0 through day 2.
7. Then the cells are transferred into the differentiation media supplemented with L-ascorbic acid 50 μg/ml and β-glycerophosphate 10 mM.
8. The media is changed every three days.
9. The gel, cell lysis, and culture medium can be harvested after culturing 14 days for analysis.

4.4.5 Neural cell culture and seeding

1. In the case of adhesion and differentiation tests, cell seeding (at a concentration of $2–8 \times 10^4$ cells/cm^2) is performed 2 days after the last mechanical dissociation in order to seed the maximum percentage of stem cells. Cells are seeded on the top surface of each assembled scaffold, where they are able to settle into the nanofiber matrices. Over time, cells migrate into the scaffold.

2. In the case of SEM imaging, cells are thoroughly mixed with 8 μl of aqueous peptide scaffold solution at a final concentration of 5–8 $\times 10^3$ cells/μl in a total final volume of 10 μl per each sample. Self-assembling is then initiated by adding basal medium slowly and placing seeded scaffolds on copper grids (Ted Pella, Inc.) at 37°C for 30 minutes. Cells are thus embedded in the scaffold.
3. For both adhesion and differentiation tests, as well as SEM imaging, cells are cultured with a medium supplemented with βFGF (10 ng/ml), added to enhance neuronal progeny differentiation. After 3 days, the medium is shifted to a medium containing a leukemia inhibitory factor (LIF, Chemicon) (20 ng/ml) and a brain-derived neurotrophic factor (BDNF, Peprotech) (20 ng/ml) to pursue the neuronal and glial population maturation in progeny. Cells are fed every three days with fresh culture medium.
4. Neural precursors isolated from the subventricular zone (SVZ) of 8-week-old CD-1 albino mice striata, at passage 10, are used. Cell proliferation is in Neurocult basal medium (mouse) (Stemcell Technologies, Vancouver), in the presence of basic fibroblast growth factor (βFGF from PeproTech, Rocky Hill, New Jersey) and epidermal growth factor (EGF from PeproTech) at final concentrations of 10 ng/ml and 20 ng/ml, respectively.
5. The medium without growth factors is used as a basal medium. Bulk cultures are generated by mechanically dissociating neurospheres and plating cells in untreated flasks at the appropriate density (1×10^4 cells/cm^2) every 4–5 days in the same growth medium.
6. Cell counting and viability examination is required at every passage, using Trypan-Blue exclusion method.

4.4.6 Preparation of MC3T3-E1 cells

Mouse preosteoblast cell line MC3T3-E1 (subclone 4) (ATCC, Virginia) can be purchased.

1. Cells are in modified medium with 10% FBS and 1.5% penicillin/streptomycin.
2. The medium is changed every 3 days.
3. When the cells become subconfluent, they are detached from the flask by treatment with aqueous solution of 0.25% trypsin for 5 minutes at 37°C.
4. The cells are subcultured at a density of 5×10^3 cells/cm^2.

4.4.7 Cell culture of human umbilical vein endothelial cells (HUVECs)

Primary isolated HUVECs are commercially available from Lonza Inc. (Walkersville, Maryland) and can be routinely grown in endothelial growth media EGM-2 (Lonza Inc.) on regular tissue culture plates. Subconfluent (~6×10^4 cells/insert) of HUVECs is seeded on the top of the scaffolds for 2D cell culture. Approximately 1×10^5 of HUVECs is suspended in 100 μl of peptide solution for 3D cell culture.

4.4.8 Cell proliferation assay

To assess the viability of cells seeded on scaffolds made of various peptides, a quantitative method, MTT assay (Sigma), was used. Four independent experiments comprising

three replicates each are required. For this viability test, the direct proportional linearity between the optical density and the viability/metabolic activity of the cell populations is assessed by verifying the linearity of 5 different standard curves at 6 increasing cell concentrations, ranging from 5×10^3 to 5×10^5 cells/well. Results are expressed as percent increase in cell population from the population seeded on day one.

Cell proliferation is determined following the manufacturer's protocols:

1. MTT (3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide) is prepared in a 5mg/ml stock solution in PBS and added to the culture medium in a ratio of 1:100.
2. Incubate at 37°C for ~60 minutes.
3. The MTT solution is removed.
4. The insoluble formazans crystals are dissolved by soaking scaffolds and cells for 15 minutes in 250 μl of dimethylsulfoxide (DMSO).
5. The absorbance at A_{550nm} is measured using a Vmax microplate reader (Molecular Devices, Sunnyvale California).

4.4.9 DNA content measurement

The number of cells on the scaffold is determined by the fluorometric quantification of the amount of cellular DNA.

1. The cell-seeded scaffold is rinsed with PBS and recovered by Na citrate buffer solution containing 50 mM Na citrate and 100 mM NaCl and stored at –80°C until assay.
2. After thawing, the cells are lysed in the Na citrate solution with occasional mixing.
3. The 10 μl of cell lysate (400 μl/insert) is mixed with Na Citrate buffer (100 μl) and DNA binding fluorescent dye solution (0.5-μl Picogreen reagent in 100-μl TE buffer).
4. The scaffolds with cells are collected for DNA purification (QIAamp DNA Mini Kit, QIAGEN). 100 μl of purified DNA sample was mixed with 100-μl DNA binding fluorescent dye solution (0.5-μl Picogreen reagent in 100-μl TE buffer, Quant-iT PicoGreen dsDNA Reagent and kits, Invitrogen).
5. The fluorescent intensity of the mixed solution is measured on a fluorescence spectrometer (Wallace Victor2, 1420 Multi-label counter, Perkin-Elmer, Massachusetts, Ex 485 nm/Em 510 nm).
6. The calibration curve between the DNA and cell number is prepared by use of cell suspensions with different cell densities.

4.4.10 Boundary-sandwiched cell migration assay

Peptide scaffolds were prepared as described earlier. Approximately 8×10^4 cells were seeded on the top of peptide scaffold A and then incubated 6 hours at 37°C with 5% CO_2 for cell attachment. The trimmed quadrate peptide scaffold A with attached cells was inversely placed on the surface of peptide scaffold B with cells between two types of scaffolds. The scaffolds with cells were cultured at 37°C for 2 days and then examined with fluorescence microscopy after nuclei staining.

4.4.11 Fluorescence microscopy

Following the experiments, cells on the hydrogels were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes at room temperature. Fluorescent Rhodamin phalloidin and SYTOX Green (Molecular Probes, Eugene, Oregon) were used for labeling F-actin and nuclei, respectively. Images were taken using a fluorescence microscope (Axiovert 25, ZEISS) or Laser Confocal Scanning Microscope (Olympus FV300).

4.4.12 Immunocytochemistry

Cell differentiation of neural lineage is assessed by double and single immunostaining with lineage-specific antibodies: nestin (1:150, Chemicon) for progenitor cells, rabbit anti-β-Tubulin (1:500, Covance) for neurons, mouse anti-Glial Fibrillary Acidic Protein (1:200, Chemicon) for astrocytes. Primary antibodies were then stained with secondary ALEXA 488 goat antimouse (1:1,000 Molecular Probes) and CY3 AffiniPure $F_{ab'}2$ antirabbit IgG antibodies (1:100 Jackson Immuno Research). Cell nuclei were counterstained with DAPI (Molecular Probes). The samples are then examined by inverted fluorescence microscope. Quantitative analyses are carried out by counting 100–300 cells for each of 10 nonoverlapping (and randomly chosen) fields. Four independent experiments comprising of five or more replicates each are analyzed.

4.4.13 SEM sample preparation

After seeding cells within the self-assembled scaffolds, cells are usually cultured between 1–14 days. Samples are prepared as follows:

1. The scaffold is soaked in 5% glutaraldehyde at 4°C for 2 hours.
2. It is then washed in MilliQ water.
3. It is then subjected to sequential dehydration steps in 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% of ethanol for 5 minutes each.
4. Samples are then placed in pressurized liquid CO_2/siphon for 1 hour using a CO_2 critical point dryer (Tousims).
5. Scaffold samples with and/or without cells are sputter-coated with gold-palladium particles (~10-nm gold coating thickness).
6. When examination the scaffold containing cells, the 3D culture systems are placed on an EM copper grid at the beginning of the experiments. The sample are prepared as steps 1–5 and examined using a JOEL JSM 6060 SEM at 2,000–100,000× magnification, 6-KV acceleration voltage, 29–32 spot size, and 12-mm electronic working distance.

4.4.14 Circular dichroism (CD)

Far-UV CD spectra are recorded between 190–260 nm at room temperature on an Aviv 62DS spectrometer.

1. All measurements were carried out in 1-mm quartz cuvette in PBS, pH 7.4. Spectra are from the accumulation of four scans. Blank spectra of the buffer without the sample are subtracted.
2. CD spectra of peptide samples at concentrations 5 μM or 10 μM in PBS are collected and compared.
3. Spectra are recorded in 1-nm steps and averaged over 2 seconds.

4.4.15 Structural study using atomic force microscopy (AFM)

1. Peptide from stock solutions (0.5%) was diluted to a working concentration of 0.01% (w/v), after a 30-minute sonication and a 2-hour stationary incubation at room temperature.
2. Atomic force microscopy (AFM) images were collected with a silicon scanning probe (FESP, Vecco Probe Inc., California) with a resonance frequency of 75 kHz, spring constant 2.8 N/m, tip curvature radius 10 nm, and 225-μm length.
3. Images are obtained with a multimode AFM microscope (Nanoscope IIIa, Vecco, Santa Barbara, California) operating in tapping mode. Typical scanning parameters were as follows: RMS amplitude before engage 1–1.2V, set point 0.7–0.9V, integral and proportional gains of 0.2–0.6 and 0.4–1.0, respectively, and scan rate 1.51 Hz.

4.4.16 Biomechanical study using rheology

The mechanical properties of the assembled hydrogels were studied with a TA Instruments AR2000 rheometer. Storage moduli were measured at frequencies from 1 to 10 rad/sec using a 20 mm, 0.5° stainless steel cone with a truncation gap of 9 μm while the strain was held constant at 1%. Each measurement was performed with 35 μl of gel assembled with 70 μl of PBS solution after allowing 1 hour for assembly.

4.4.17 Alkaline phosphatase (ALP) staining for MC3T3-E1 cells

ALP staining is conducted using an Alkaline Phosphatase Staining Kit (85L-2, Sigma, Missouri) according to the manufacturer's protocol.

1. Cells on the scaffold in the inserts are washed twice in saline, fixed for 6 minutes at room temperature in citrate fixative solution contained in the kit.
2. Wash using Mill-Q water. AS-MX solution in the kit is added to the insert and incubated at room temp for 30 minutes.
3. The scaffold in insert is rinsed well using Mill-Q water three to four times and the cells are observed under a light microscope.

4.4.18 Biochemical assays for alkaline phosphatase (ALP) activity for MC3T3-E1 cells

ALP activity in the cells on hydrogel is determined by fluorometric quantification.

1. The cell-seeded hydrogel scaffold is rinsed with saline and recovered by ALP lysis buffer containing 2 mM $MgCl_2$ and 0.05% Triton X-100 and then stored with at –80°C until assay.

2. After thawing, the cells are lysed in the ALP lysis solution with occasional mixing. The 20 μl of cell lysate (300 μl/insert) is mixed with 20-μl $MgCl_2$ solution and 150-μl fluorescent assay buffer, both provided in the Alkaline Phosphatase Fluorescence Assay Kit (Sigma, Missouri).
3. Then 1 μl of suspended substrate (4-methylumbelliferyl phosphate, 10 mM) is added and incubation occurred at room temperature for 1 hour.
4. The fluorescent intensity of the mixed solution is measured on a fluorescence spectrometer (Wallace Victor-2, 1420 Multi-label counter, Perkin-Elmer, Massachusetts, Excitation 355 nm-Emission 460 nm).
5. The calibration curve between the ALP activity and fluorescent is prepared by use of alkaline phosphatase (control enzyme) with different concentration.

4.4.19 Low protein release from the peptide scaffold

In order to combine protein in the scaffold that stimulate cell proliferation, differentiation and migration, we develop method for slow release the proteins.

Peptide hydrogel scaffold is formed using well-established protocols (see Sections 4.1 and 4.2).

1. The Ac-$(RADA)_4$-$CONH_2$ peptide solution or other functionalized sapeptides is mixed with phosphate-buffered saline (PBS, pH7.4) containing protein at a final concentration of 5 nM and 5 μM, respectively.
2. An aliquot 40 μl of the solution is transferred into the wells of a 384-well plate and gelation occurred within 15 minutes.
3. Subsequently, 70 μl of PBS was slowly added to the 40 μl of the hydrogel, so as to satisfy the perfect-sink conditions and allow for the determination of the protein release profile.
4. Another aliquot 40 μl of the supernatant is replaced with the same volume of fresh PBS at frequent time points. The hydrogel volume does not change and, therefore, protein release is not attributed to hydrogel degradation or swelling.

4.5 Data Acquisition, Results, and Interpretation

4.5.1 Designer self-assembling peptide nanofiber hydrogel scaffold

We have shown that this matrix encapsulates several cell types in a manner similar to the extracellular matrix, the peptide hydrogel scaffold system can be used for 3D cell culture, slow protein release, tissue engineering, and tissue regeneration applications.

A single molecule of the ionic self-complementary peptide RADA16-I is shown in Figure 4.5. Millions of peptide molecules self-assembled into individual nanofibers that further form the nanofiber scaffold (Figure 4.5). The nanopores range from a few nanometers to a few hundred nanometers; the scales are similar in size to most biomolecules. The scaffold hydrogel is completely transparent, which is a very important requirement for accurate image collections for uses in 3D tissue cell cultures.

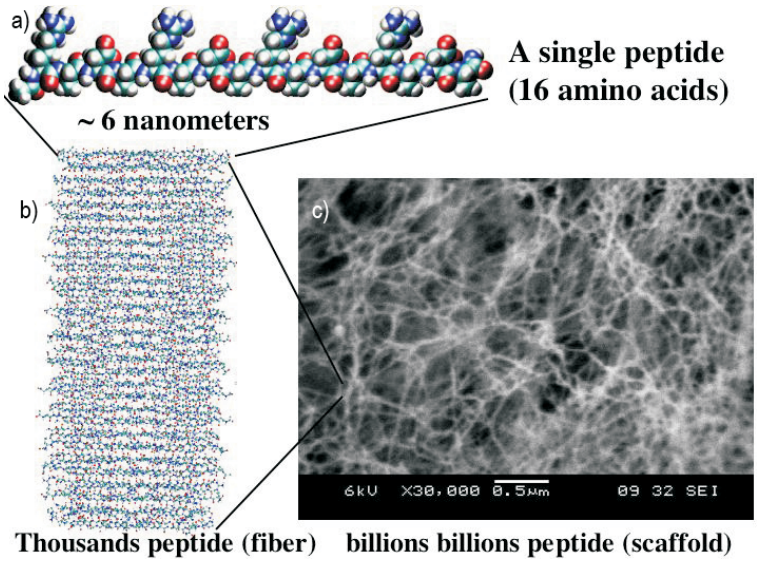

Figure 4.5 Self-assembling peptide RADA16-I nanofiber scaffold hydrogel. (a) Amino acid sequence of RADA16-I, molecular model of a single RADA16-I nanofiber—the dimensions are ~6 nm long, 1.3 nm wide, and 0.8 nm thick; (b) hundreds of thousands of individual peptides self-assemble into a nanofiber; and (c) SEM images of RADA16-I nanofiber scaffold. Note the scale bar, 0.5 μm or 500 nm.

4.5.2 3D cell cultures

The designer self-assembling peptide nanofiber scaffolds have been shown to be an excellent biological material for 3D cell cultures (Figure 4.6), not only capable of stimulating stem cell differentiation [5] and cell migration into the scaffold [6, 7] but also for repairing tissue defects in animals (results not shown).

We developed several peptide nanofiber scaffolds designed specifically for osteoblasts [6]. 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 sequence PGR (PRGDSGYRGDS).

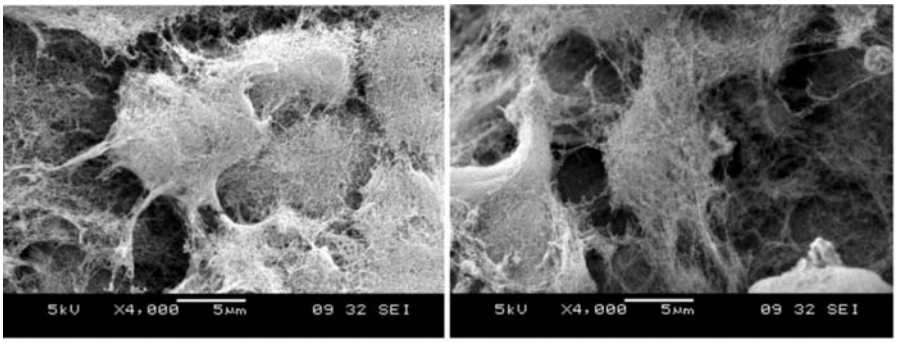

Figure 4.6 Mouse adult neural stem cells are embedded in designer self-assembling peptide scaffold. Note the cells are totally surrounded by the scaffolds and interact directly with both the peptide nanofibers and extracellular matrix that share similar scales, which are indistinguishable based on their sizes. This microenvironment is closer to the reality in the body.

The bioactive peptide scaffolds is made by mixing the pure RADA16-I and designer peptide solutions together. The molecular integration of the mixed nanofiber scaffolds is examined using AFM. Compared to pure RADA16-I scaffold, these designer peptide scaffolds significantly promote 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, thus demonstrating that the designer, self-assembling peptide scaffolds promoted the proliferation and osteogenic differentiation of MC3T3-E1. Under the identical culture medium condition, confocal images showed that the designer PRG peptide scaffold stimulated cell migration into the 3D scaffold (Figure 4.7) [6]. Without the modified motif, cells did not migrate in 3D.

4.5.3 Cell migration in peptide scaffolds

In order to study the preferences of endothelial cells' migration in response to the components of surrounded scaffolds and the importance of functional peptide motif for guiding cells migration, we designed the clear-boundary sandwich assays to assess endothelial cell migration between two types of peptide scaffolds. As shown in Figure 4.8(b), endothelial cells plated on the scaffolds RAD/PRG [Figure 4.8(b), parts a and e] and the RAD/KLT [Figure 4.8(b), parts b and f] settled in these scaffolds and no visible migration towards the RADA16-I scaffold was observed. In contrast, cells seeded on RADA16-I scaffold directionally migrated towards functionalized peptide scaffolds [Figure 4.8(b), parts c, d, g, and h]. These experiments unambiguously show the biological importance of the designer functionalized peptide scaffolds that induce cell directional migrations. This is a very important observation since there are no additional soluble growth factors in these scaffolds. These observations suggest the adhesion peptide motif alone can induce cell migrations without extract-soluble factors.

Many factors are involved in cell unidirectional migration, such as soluble chemoattractants and attachments on the surrounding matrix. In literature reports, endothelial cell migration is mostly stimulated by growth factors such as VEGF and bFGF, and also is activated in response to integrins binding to the ECM component. Our results demonstrated unequivocally that the adhesive interactions governed mainly by the functional motif of PRG alone promote cell attachment and migration from

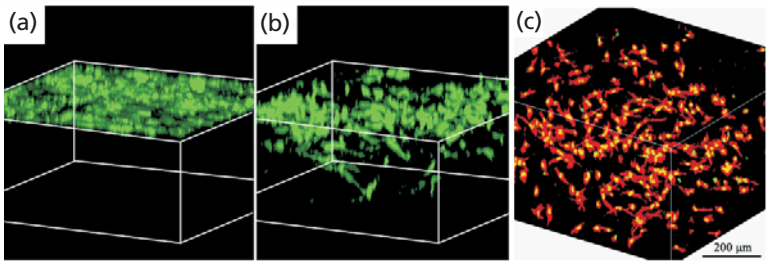

Figure 4.7 Reconstructed image of 3D confocal microscopic image of cells culturing on the different scaffolds consisting of different mix ratio of RADA16 1% (w/v) and PRG 1% (w/v) using calcein-AM staining. (a) 1-part PRG: 9-parts RADA16. (b) 7-part PRG: 3-part RADA16. In the case of 1-part PRG scaffold, the cells attach on the surface of the scaffold, whereas in the case of 7-part PRG scaffold, the cells migrate ~300–400 μm into the scaffold. There is a drastic cell migration into the scaffold with a higher concentration of PRG motif. (c) Endothelial cell morphologies in 3D peptide RADA16/PRG scaffolds. It is evident that cells migrate ~400–500 μm into the scaffold.

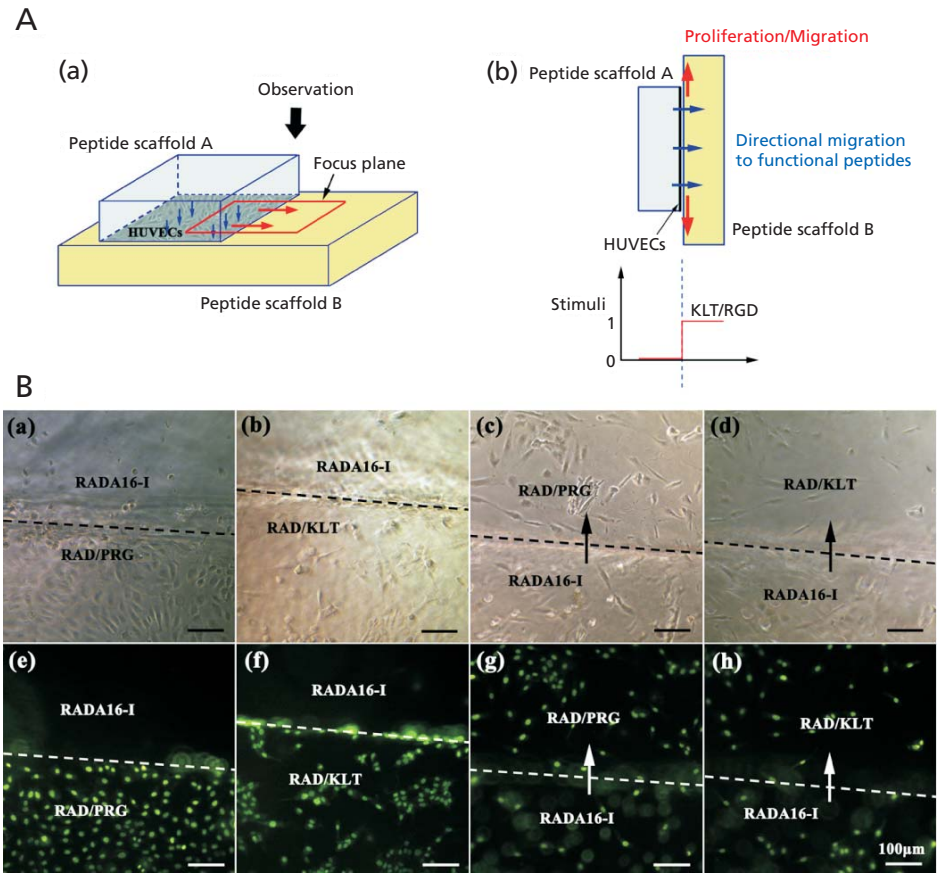

Figure 4.8 Endothelial cell unidirectional migration in response to functional peptide scaffolds. (a) Schematic illustrations of cell directional migration. a. Clear-boundary-sandwich cell migration assay. b. Directional migration induced by functional motifs. (b) Phase contrast microscopy images of HUVECs seeded on peptide scaffolds: a. RAD/PRG; b. RAD/KLT; c. and d. RADA16-I, and fluorescent SYTOX Green nuclear staining for e. RAD/PRG; f. RAD/KLT; g. and h. RADA16-I. Cells directionally migrated from RADA16-I to RAD/PRG (c and g) and RAD/KLT (d and h). The scale bar is 100 μm for all panels.

RADA16-I to RAD/PRG scaffold, whereas the VEGF mimicking sequence of KLT may mainly serve the similar function of VEGF to regulate endothelial cell migration. Cells seeded on RADA16-I were probably induced by the functional motifs of PRG and KLT and migrated to/on these functionalized peptide mixtures scaffolds [Figure 4.8(a), part b). More systematic experiments will be carried out to further address these questions.

4.5.4 Rheology of peptide hydrogel scaffold

Rheology was used as a method for comparison of the relative mechanical stiffness of the assembled peptides. The storage moduli (G′) were measured at low frequencies where the loss moduli (G′′) were too low to be of consequence. Therefore, the average G′ over the frequency range of 1 to 10 radians/sec is used to compare the relative stiffness of the scaffolds. This information is useful in describing the type of mechanical environment that seeded cells are presented with and can serve as a macroscopic measure of the extent of interactions formed between assembled peptides. The functionalized peptides

were generally found to exhibit reduced storage moduli when compared to RADA16-I (G' = 1,630 Pa), however, peptide RADA16-GGFLGFPT (G' = 2,320 Pa) showed an increase in stiffness. This is perhaps due to the relatively high degree of hydrophobicity of the added motif (illustrated in Figure 4.3). Peptides of greatest interest for neural stem cell cultures, RADA16-GGSKPPGTSS (G' = 762 Pa) and RADA16-GGPFSSTKT (G' = 53.5 Pa), show reduced storage moduli in comparison with RADA16. All of the assembled peptides exhibit relatively low storage moduli in comparison with Matrigel (G'= 5,408 Pa), which was used as a positive control in the cell culture studies. The time required for the self-assembly process to occur was also examined through rheological analysis. Peptide RADA16-GGPFSSTKT was found to have a fairly linear increase in storage modulus with time for approximately one hour, at which point the G' values stabilized at 762 Pa.

4.5.5 Tissue regeneration and tissue engineering

The importance of nanoscale becomes obvious in 3D cell culture. It is clearly visible in the images that the cells are embedded in the self-assembling peptide nanofiber biological scaffolds in the truly 3D culture (Figure 4.9). Here, the cells and cell clusters intimately interact with the extracellular matrix cells that they produce. Since the scaffolds are made mostly of water, ~99% water at 1% peptide solid, cells can migrate freely without hindrance, similar to fish swimming freely in a seaweed forest in the sea.

These self-assembling peptide nanofiber biological scaffolds have become increasingly important not only in studying the spatial behaviors of cells, but also in developing approaches for a wide range of innovative medical technologies including regenerative medicine (Figure 4.9). Some examples include: neurite growth and differentiation, neural stem cell differentiation, cardiac myocytes, bones, cartilage cells, human umbilical vein endothelial cells, and keratinocytes cultures. The peptide scaffolds from RADA16-I and RADA16-II forms a nanofiber scaffold in physiological solutions, which stimulated extensive rat neurite outgrowth and successfully achieved active synapse formation on the peptide scaffold [3].

4.5.6 Protein releases from the peptide nanofiber hydrogel scaffold

In order to effectively improve long-term cell cultures, we also developed a method to embed a wide spectrum of proteins within the peptide scaffolds, allowing them to release slowly. This method is not only useful for 3D cell culture, but also for delivering a wide range of therapeutic protein medicine including enzymes, growth factors, and monoclonal antibodies.

We have demonstrate that the Ac-$(RADA)_4$-$CONH_2$ peptide hydrogel is an efficient slow delivery carrier not only for small molecules, but also for a variety of proteins including lysozyme, trypsin inhibitor, BSA, and IgG with differing physicochemical properties (pI4.6 – pI8.5, MW 14.3 kDa – 146 kDa) and morphologies, which are encapsulated within the Ac-$(RADA)_4$-$CONH_2$ peptide hydrogel (Figure 4.10).

It is crucial to determine if the processes involved in incorporating and releasing proteins from the peptide hydrogel adversely affect their conformation and function. To this end, the released protein structure was analyzed using circular dichroism (CD) and fluorescent spectroscopy and bioassays were conducted to verify protein functionality. The presentation of functional proteins and the elucidation of crucial protein-hydrogel

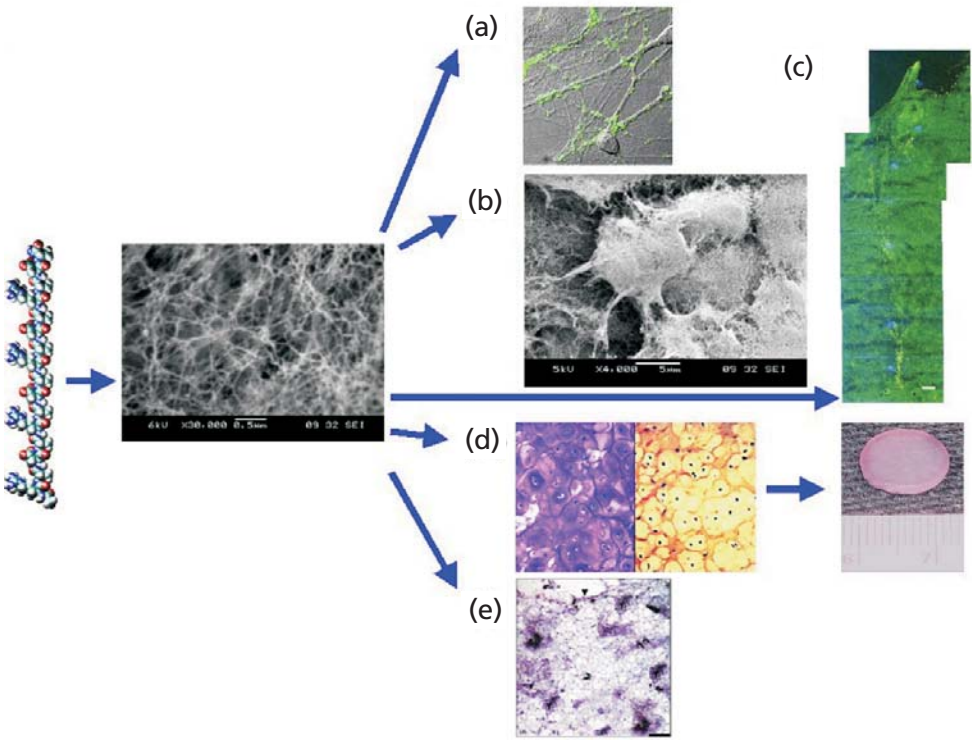

Figure 4.9 From designer peptides to scaffolds to tissues. (a) Active synapses on the peptide surface. Primary rat hippocampal neurons form active synapses on peptide scaffolds. The confocal images show bright discrete green dot labeling indicative of synaptically active membranes after incubation of neurons with the fluorescent lipophilic probe FM-143. FM-143 can selectively trace synaptic vesicle turnover during the process of synaptic transmission. The active synapses on the peptide scaffold are fully functional, indicating that the peptide scaffold is a permissible material for neurite outgrowth and active synapse formation. (b) Adult mouse neural stem cells embedded in 3D scaffold. (c) Brain damage repair in a hamster. The peptide scaffold is injected into the optical nerve area of brain that is first severed with a knife. The injury site is sealed by the migrating neural cells after two days. A great number of neurons form synapses (image courtesy of Rutledge Ellis-Behnke). (d) Peptide KLD12 (KLDLKLDLKLDL), chondrocytes in the peptide scaffold, and cartilage. The chondrocytes stained with TB show abundant GAG production (left panel) and the antibody to type II collagen demonstrates abundant type II collagen production (right panel). A piece of premolded cartilage with encapsulated chondrocytes in the peptide nanofiber scaffold. The cartilage formed over a 3–4-week period after the initial seeding of the chondrocytes (image courtesy of John Kisiday). (e) Von Kossa staining showing transverse sections of primary osteoblast cells on the HA-PHP-RADA16-I self-assembling peptide nanofiber scaffold. Scale bar = 0.1 mm. The intensely stained black areas represent bone nodules forming. (Image courtesy of Maria Bokhari.)

events are considered as significant advances that are required for furthering designer peptide nanofiber hydrogel scaffold for various nanomedicine applications.

An efficient controlled release system should yield biologically active proteins. We tested the released proteins and found that encapsulation and release do not affect the secondary or tertiary structure of the proteins studied, nor is their functionality diminished. These results lay the foundation for creating new tailor-made peptide hydrogels for controlled release of proteins.

Peptide hydrogel scaffolds can be easily designed and synthesized to control the release of proteins and other therapeutic compounds by varying the density of the

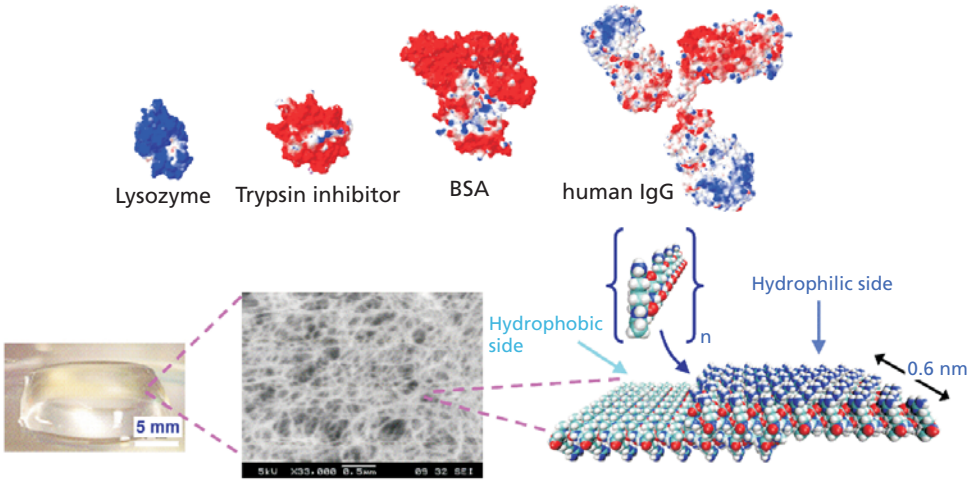

Figure 4.10 Molecular size models of lysozyme, trypsin inhibitor, BSA, and IgG as well as of the Ac-(RADA)$_4$-CONH$_2$ peptide monomer and of the peptide nanofiber. Color scheme for proteins and peptides: positively charged (blue), negatively charged (red), hydrophobic (light blue). Protein models were based on known crystal structures.

nanofibers of the hydrogel or by changing the charge of the nanofibers simply by adding amino acids with positively or negatively charged side groups. Overall, this system would allow the release of diffusing molecules in a sustainable and highly efficient way. We anticipate that further fine-tuned systems will have a wide range of applications in biomedical technology and clinical medicine.

It is possible to combine the 3D culture system with the slow releases of both small molecules and proteins to forge a powerful technology not only for effective 3D cell culture but also for accelerating tissue regeneration.

4.6 Discussions and Commentary

Flat glass and plastic surfaces are not representative of the real cellular environment of living organisms. Tissue-specific architectures, mechanical forces, and cell-cell interactions are usually not represented in 2D inert surfaces. Monolayer cell cultures often modify the cellular intrinsic signal pathways, thus 2D studies are not realistic and result in flawed models, which deviate from the conditions found in tissues where cells interact through chemical and mechanical stimuli with adjacent cells and with extracellular matrix.

Biologically derived biomaterials such as Matrigel from the mouse sarcoma and different types of collagen have been used as a template for 3D cell studies. However, their biological source not only prevents them from any applications in humans but also results in studies, which are not reproducible because their composition may vary from lot to lot. Furthermore, the fact that they contain residual growth factors and other nonquantified impurities results in ambiguous conclusions.

The biologically inspired nanoscale designer self-assembling peptide scaffolds mimic the in vivo environment and serves as permissive substrates for cell growth, differentia-

tion, and biological function. Self assembling peptide nanofiber scaffolds are useful not only for furthering our understanding of cell biology in 3D environment, but also for advancing medical technology, tissue engineering, regenerative biology, and medicine. The self-assembling peptide scaffolds are inert, nonimmunogenic, and nontoxic, consisting of natural amino acids. Peptide hydrogel scaffolds are increasingly important not only in studying the cell behavior in a realistic 3D environment, but also in developing innovative technologies in biomedicine.

Troubleshooting Table

Problem	Explanation	Potential Solutions
Self-assembling peptide solution is viscous.	Limited peptide association is possible during storage.	Ultrasonication facilitates peptide nanofiber dissociation and enables easy pipetting.
Hydrogel is very soft.	The peptide nanofiber density is probably too low; more nanofibers are required to reinforce the hydrogel structure.	Increase the concentration of self assembling peptides; increase the salt concentration of the medium to at least 100 mM.
	The electrolyte concentration may be low resulting in incomplete assembly.	
Cells do not migrate.	The peptide nanofiber density is too high preventing cell migration.	Use lower density peptide hydrogel scaffolds.
Cell do not proliferate.	The peptide nanofiber density is too high and the encapsulated cells cannot grow and proliferate.	Lower density gel is required.
Hydrogel appears fragmented.	The self assembly process which leads to sol-gel transition is not complete.	Allow at least 15 minutes to complete the sol-gel transformation.
	The peptide hydrogel is a soft biomaterial with elastic properties; however, excessive force will cause macroscopic ruptures.	Avoid contact of the gel surface with the pipette tips; change medium gently.
Poor quality microscope images.	Tissue cell cultures in the peptide hydrogel scaffold result in 3D morphologies where focusing may be difficult.	3D tissue culture imaging is facilitated by using confocal microscope techniques.
Poor cell immunostaining.	Staining of the cells requires sufficient time for the antibodies and other fluorescent probes to diffuse through the peptide hydrogel and interact with the cells.	Longer incubation times are necessary to allow for the PFA and antibodies to penetrate the gel and interact with cells.

4.7 Applications Notes

The sol-gel transformation of the self-peptides occurs in response to external stimuli when the individual peptide monomers are brought in contact with an electrolyte solution having a composition similar to that of most biological fluids. This harmless triggered self-assembling and the fact that sapeptide hydrogel scaffolds are biocompatible, nontoxic, and nonimmunogenic are the major advantages of this nanotechnology inspired system and allow for numerous applications in bioengineering via a number of different methods.

4.7.1 In vivo injectable self-assembling peptides

It has been shown that injection of the peptide solution in the damaged tissue may be used for regeneration of the heart muscle and of the optical tract in mice as well as for wound healing and hemostasis. In these cases, the self-assembling peptide solution alone or mixed with progenitor cells and growth factors was delivered through a small

diameter needle in the injured spot. This methodology may be applied in many in vivo tissue regeneration applications of internal organs where the site of injury is focalized and where a minimally invasive treatment is required.

The RADA16-I self-assembling peptide has been used for neural regeneration in optic nerve lesions. Hamster pups with an injured optical tract are treated by injecting RADA16-I self-assembling peptides into the wound. Control animals with the same brain lesion were treated with an injection of isotonic saline solution in the injured tissue. Animals were sacrificed at various time frames for brain examinations where it was shown that animals treated with self-assembling peptide scaffold the brain tissue was healed and new axons connecting to the retina were grown beyond the tissue lesion. In all animals treated with the peptide scaffold, a significant functional improvement in their sight capabilities was observed using specifically developed behavioral tests.

4.7.2 In vitro multicell system for tissue engineering

Although significant advances have been made in the field, the final goal to engineer and form a tissue in vitro has not been reached yet. Many studies using the self-assembling peptide nanofiber hydrogel scaffold can support the proliferation of numerous mammalian cells including endothelial cells for angiogenesis applications and tissue vascularization, neural stem cells for neural tissue regeneration, mesenchymal stem cells for bone regeneration, chondrocytes for cartilage regeneration, fibroblasts for wound healing and skin graft therapies, and cardiac myocytes for heart muscle regeneration. Hydrogel scaffolds porosity ranges from 100 nm to 500 nm, and the scaffold mechanical properties matches with biomechanical requirements necessary for cells to branch, differentiate, migrate, and establish new functional cytoarchitectures inside the scaffold matrix. This may allow for in vitro formation of a functional, fully vascularized tissue, which may then be transplanted into the host.

4.7.3 Mixed peptide hydrogel with polymer composites

Self-assembling peptides may be used alone or mixed with known compatible biopolymers to generate novel materials where the biocompatibility of the peptide hydrogel is combined with the mechanical properties of polymers. This may be achieved simply by mixing the two components so as to integrate the peptide hydrogel within the volume of the stiff polymer, or by developing advanced hybrid peptide-polymer materials as in the case of the peptide-polymer conjugates. Both of these approaches result in the formation of peptide-based materials with increased mechanical properties that can be used for implantation in the reconstruction of hard tissues such as bone and cartilage.

The possibilities are limitless, thus offering numerous alternative strategies for generating tailor-made biomaterials for biomedical applications with desired properties and characteristics.

4.8 Summary Points

1. The 3D cell culture studies are advantageous over the traditional less reliable 2D studies where cells are cultured on the flat surface of a plastic or glass material.

2. Designer self-assembling peptides are nontoxic, nonimmunogenic, and biocompatible, while the degradation products are natural amino acids.
3. The peptide assembly process into nanofibers occurs in the presence of an electrolyte solution or biological fluids.
4. Designer self-assembling peptide hydrogel scaffolds support cell growth, proliferation, migration, and differentiation of a number of cell types and can be used for tissue engineering and tissue regeneration applications.
5. The new generation of designer self-assembling peptides consists of active peptide motifs that carry cell adhesion and cell differentiation activities; thus, they can be fairly considered as more efficient scaffolds for both in vitro cell culture and in vivo tissue regeneration.

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Supplementary Electronic Materials and Resources

2D coating and 3D peptide scaffold multiwell cultures
Cell recovery from the peptide scaffold
ECM additive to the peptide scaffold
Insert culture system preparation for peptide scaffold
In vivo injection of the peptide scaffold for animal tests