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Designer Self-Assembling Peptide Nanofiber Scaffolds for Study of 3-D Cell Biology and Beyond

Dedicated to George and Eva Klein 80th Birthday Symposium

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"You should always ask questions, the bigger the better. If you ask big questions, you get big answers."

Francis Crick (1916–2004)

Biomedical researchers have become increasingly aware of the limitations of the conventional 2-D tissue cell cultures where most tissue cell studies including cancer and tumor cells have been carried out. They are now searching and testing 3-D cell culture systems, something between a petri dish and a mouse. The important implications of 3-D tissue cell cultures for basic cell biology, tumor biology, high-content drug screening, and regenerative medicine and beyond are far-reaching. How can nanobiotechnology truly advance the traditional cell, tumor, and cancer biology? Why nano is important in biomedical research and medical science? A nanometer is 1000 times smaller than a micrometer, but why it matters in biology? This chapter addresses these questions. It has become more and more apparent that 3-D cell culture offers a more realistic local environment through the nanofiber scaffolds where the functional

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Eva Klein

George Klein

Ingemar Ernberg Klas Kärre, Marie Henriksson, Maria Masucci are at the George and Eva Symposium, June 2005.

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properties of cells can be observed and manipulated. A new class of designer self-assembling peptide nanofiber scaffolds now provides an ideal alternative system. Time has come to address the 3-D 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. © 2008 Elsevier Inc.

PROLOGUE

Professors George and Eva Klein not only have inspired a few generations of leading biomedical researchers but also have had enormous influence in the fields of tumor and cancer biology, immunology, and virology around the world. They personally taught and mentored a very large number of influential biomedical and medical researchers and medical scientists not only at the Karolinska Institute, Stockholm, Sweden, but also in Europe and rest of the world. In addition to their pioneer research, either intentionally relentless pursuit or quickly recognizing the unexpected discoveries, they have had profound impact beyond their own fields; their contributions have also been felt far beyond the defined disciplines. Furthermore, because of their warm and open personalities, tireless traveling, keen interest in science, medicine and culture, as well as their impatience and intolerance for fools, their names have become synonymous with extremely high scientific standards and they have made a wide spectrum of friends, become close colleagues, and got to know a large number of acquaintances. They are highly respected beyond any geographic location, from Europe, Middle East, North and South America to China. Their names are legendary, and their legacy will have a lasting impact on many generations of scientists to come.

One might wonder how could a young researcher from China, outside of tumor and cancer biology, immunology, and virology fields, become very close to George and Eva Klein? The answer lies, not surprisingly, in their warm and open personality. Here is a moving story that illustrates the personality of George and Eva Klein.

When I was a graduate student studying *Tetrahymena* genetics at the University of California, Santa Barbara, in 1987, I never heard of George and Eva Klein. Although I have read literature widely, my own interest was in the detail structure of DNA, particularly the left-handed Z-DNA discovered by Alexander Rich and his colleagues at MIT in 1979. My interest was not only a scientific one but also a philosophical one. I asked why nature sometimes has remarkable symmetry and sometimes does not. Why most helices at molecular scales, for example, alpha-helix in proteins, DNA and RNA double helix in nucleic acids, and some helices in polysacchrides, are mostly right-handed.

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My exposure to virology and immunology was kept at minimal, although I took two courses in these subjects. But in both the courses, the complex names, both in English and in non-English, as well as the endless abbreviated terms, Latin names, and hard-to-remember acronyms made these subjects less attractive as I was still struggling to learn English.

Then things changed dramatically in September 1987, when my son, Niklas, who had just turned 3, was diagnosed with childhood acute lymphoblast leukemia (ALL) with ~68% leukemia cells with double chromosomal translocations (7:9; 6:21) in his bone marrow. The doctors at several oncology clinics and at the Children's Hospital of Los Angeles refused to give a prognosis. This was devastating! Then I instantly read the latest literature about chromosomal translocations and their relationship in childhood leukemia. George Klein's name came up many times. At that time, I had never read or heard George Klein's name before, and had no idea that he is one of the most prominent tumor and cancer biologists and an authority on chromosomal translocation in the world. I wrote him asking for help for what was the most available treatment at that time. I wrote, with my cryptic English, to about 30 people who seem to be experts on childhood leukemia.

Not surprisingly, most people did not reply. Only three people replied to a totally unknown Chinese student, pleading for help; among them were Sharon Murphy, then at St. Jude Hospital; Janet Rowley of University of Chicago; and George Klein of Karolinska Institute, Sweden. George Klein not only wrote me a letter but also sent me a big package of his publications relevant to chromosomal translocation and other tumor biology. George probably does not remember my request anymore since he routinely replies requests, large and small. This is my first encounter with George Klein, not as a scientist but as a father trying to find the cause and cure for my son's mysterious disease.

Many years later, when I first visited Karolinska Institute in September 1999 to attend Dr. Bian Zhao's Ph.D. thesis defense, I met George Klein in person for the first time. I had no formal appointment with him nor had telephoned him in advance. I just walked into his office while he was very busy. However, he received me and had a chat with me. He not only signed his book *Atheist and Holy City*, recommended to me by Robert Horvitz, but also gave me another of his book, *Living Now*. He was very generous with his time and very kind to a stranger of foreign origin.

Subsequently, I invited George Klein to give a History of Biology Lecture at Massachusetts Institute of Technology (MIT) in September 2000. When I asked George to give me a title of his lecture, he did not just give me one but four titles! He came together with his son Dr. Peter Klein, a mathematician with a Ph.D. from Columbia University, New York. Peter was then interested in complex problems involving mathematics in biology. George not only met his old friends but also made new friends during his visit at MIT. Later

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when I visited Peter in his home in Greenwich near New York City. I had many visits with Peter Klein in his home and elsewhere since his daughter and son are about the same age as my son, Niklas. Au3

I met Eva Klein in Peter's house in November 2002. Eva in her usual open-minded, direct, and warm manner immediately told me that I am now an honorary member of her family since I am of the same age as Peter Klein. I am very honored. During conversation, I learned that Eva is the person who, together with her postdoc, discovered the now extremely important and ubiquitous Natural Killer cells, a crucial advancement for immunology and tumor biology. I asked her why she gave the name Natural Killer cells. She told me that these cells were discovered from the control experiments and since these cells could be activated without external stimulations, they are the natural killers. Since this discovery was mostly either taken for granted or totally forgotten, I decided to invite Eva Klein to give a talk on the subject to inspire young researchers to make more discoveries, to do good controls, to make very careful observations, and to question unexpected results.

I also arranged Eva Klein to give the same History of Biology Lecture Series at MIT on March 24, 2003. She gave me a very unusual title: *Natural Killer cells: An unexpected discovery (met first as an annoying phenomenon)*. Her lecture was very well received. Robert Horvitz, Richard Hynes, Jack Buchanan, Gobind Khorana, Boris Magasanik, and many other faculty and students from the MIT Biology Department and elsewhere attended her lecture, full of interesting stories and current advancement of NK cells. She later told me that my insistence on her to give a lecture on the history of the discovery of NK cells has encouraged her to look into the active field much more closely.

Although their research areas are in tumor and cancer biology, virology, and immunology, they both recognize the importance of new findings outside their fields immediately. After my lecture, "Beyond Petri Dish," hosted by the late colleague and friend, structural biologist, Carl Brändén at Karolinska Institute in April 2003, Eva immediately asked me to edit a special focus volume for *Seminar on Cancer Biology* on 3-D cell culture. The issue came out in October 2005. George, likewise, suggested many experiments to study cancers in the designer biological scaffolds in 3-D systems.

Both George and Eva Klein have very little patience to tolerate fools. They are outspoken for many issues, including science, culture, and politics. George has written many books that are utterly refreshing. The ideas expressed and topics selected in his books are direct, sharp, intelligent, lucid, and eloquent. I wish there were more people who could write as direct as George Klein. Interestingly, he does not per se write the books; rather, he dictates his books, a special ability. When I stayed in their home, I found one morning, ~6 o'clock, he was busy dictating another book.

Both George and Eva survived the horrible holocaust, so they do not waste time for unimportant things. They are extraordinary people, both as

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scientists and as humanists. They always ask big questions in medical science and other matters they consider important. They have made an enormous contribution not only to biomedical research but also for enriching our culture and politics. They are truly rare world-class citizens.

I. INTRODUCTION

Nearly all tissue cells are embedded in a 3-dimensional (3-D) microenvironment in the body surrounded by nanoscale extracellular matrix. On the other hand, nearly all tissue cells, including most cancer and tumor cells, have been studied in 2-dimensional (2-D) petri dish, 2-D multiwell plates, or 2-D glass slides coated with various substrata. How can one reconcile the apparent disparity? Likewise, although millions of cell biology papers have been published using the 2-D culture systems, one must ask how we can be so certain that the results obtained from the 2-D system truly reflect the *in vivo* conditions. Science, after all, is to constantly ask questions, big and small.

II. 2-D OR NOT 2-D

Although petri dish has had an enormous impact on modern biology, the petri dish culture system, including multiwell plates, glass cover slips, etc, is less than ideal to study tissue cells for several reasons: (1) It is a 2-D system that is in sharp contrast to the 3-D environment of natural tissues both in animals and in plants. (2) The petri dish surface without coating is rigid and inert, again in sharp contrast to the *in vivo* soft environment where cells intimately interact with the extracellular matrix and with each other. (3) The tissue cell monolayers on coated 2-D surface, such as poly-L-lysine, collagen gels, fibronectin, laminin and Matrigel (Kleinman and Martin, 2005; Kleinman *et al.*, 1986) as well as other synthetic materials containing segments of adhesion motifs, have only part of the cell surface attached to the materials and interact with neighboring cells. Often, the remaining parts are directly exposed to the culture media, unlike the tissue environment where every cell intimately interact with its neighbor cells and the extracellular matrix. Thus 3-D-matrix interactions display enhanced cell biological activities and narrowed integrin usage. (4) The transport phenomena of 2-D and 3-D are drastically different. In 2-D 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. (5) Cells cultured on a 2-D 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,

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cell collections using trypsinization or mechanically using rubber policeman may have an adverse effect on cell–materials/environment interactions. In contrast, cells cultured on 3-D scaffolds are more readily transportable without significantly harming cell–material and cell–cell interactions, thus providing a significantly new way to study cell biology.

III. MICRO- AND NANOSCALES, WHY THEY ARE IMPORTANT?

The importance of length scales is apparent, for example, the scales of trees and grasses (Fig. 1). Both are made of the same basic building blocks, sugars that are polymerized by enzymes to produce cellulose fibers. Trees, usually 20–30 cm in diameter, are common in forests. If animals are in the forest, they can either go between the trees or climb onto the trees, they cannot go through the trees because they are in similar scales as the trees. On the other hand, grasses are usually 0.5 cm in diameter; grasses 0.3–1 cm in diameter are common. Although animals are embedded and surrounded in in grasses, they can move freely in the grasses. This analogy can be directly extended to scaffolds in various scales.

In the past three decades, several biopolymers, including PLLA, PLGA, PLLA-PLGA copolymers, and other biomaterials including alginate, agarose, collagen gels, and others, have been developed to culture cells in 3-D (Atala and Lanza, 2001; Hoffman, 2002; Lanza *et al.*, 2000; Palsson *et al.*, 2003; Yannas, 2001). These culture systems have significantly advanced our understanding of cell–material interactions and fostered a new field of tissue engineering and regenerative medicine. Attempts have been made to culture cells in 3-D using synthetic polymers or copolymers. However, these synthetic polymers are often processed into microfibers, $\sim$10–50 μm in diameter, that are similar in size to most cells ($\sim$5–20 μm in diameter). Thus, cells attached to microfibers are still in a 2-D environment with a curvature depending on the diameter of the microfibers. Therefore, cells attached to microfibers are in fact in 2-D despite the various curvatures associated with the large diameter microfibers. Furthermore, the micropores ($\sim$10–200 μm cross) between the microfibers are often $\sim$1000–10,000-fold greater than the size of bimolecular, including vitamins, amino acids, nutrients, proteins, or drugs, which as a consequence can quickly diffuse away, much like a car driving on highways. For a true 3-D environment, a scaffold's fibers and pores must be substantially smaller than the cells. In order to culture tissue cells in a truly 3-D microenvironment, the fibers must be significantly smaller than cells so that the cells are surrounded by the scaffold, similar to the extracellular environment and native extracellular matrices

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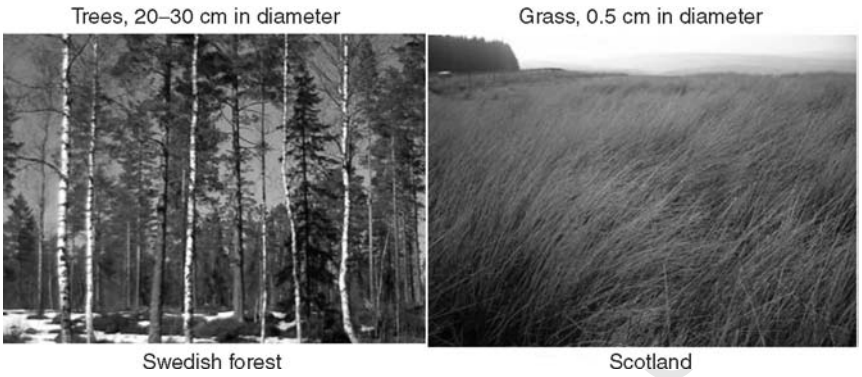

Fig. 1 The drastic difference in scales. Both trees and grasses are made of cellulose, or the same building blocks—sugars, but with very different scales. The trees shown on the left are 20–30 cm in diameter and the distance between the trees is in tens of meters. Animals cannot walk through the trees but between them. Some animals can climb on the trees (left panel). This is in analogy; cells ~5–20 μm can only attach to the microfibers. On the other hand, each grass is about 0.5 cm in diameter. When animals walk in the grass field, they are fully surrounded by the grasses, which do not hinder their movement. In this case, animals are embedded in 3-D (right panel). In analogy, cells in nanofibers are fully embedded in the nanofiber scaffolds, where they can still move freely without hindrance. (See Color Insert.)

(Ayad *et al.*, 1998; Kleinman *et al.*, 1986; Kreis *et al.*, 1999; Lee *et al.*, 1985; Oliver *et al.*, 1987; Timpl *et al.*, 1979).

Animal-derived biomaterials (e.g., collagen gels, polyglycosaminoglycans, and Matrigel) have been used as an alternative to synthetic scaffolds (Bissell *et al.*, 2002; Cukierman *et al.*, 2001, 2002; Kleinman *et al.*, 1986; Kubota *et al.*, 1981; Lee *et al.*, 1985; Oliver *et al.*, 1987; Schmeichel and Bissell, 2003; Weaver *et al.*, 1995; Zhau *et al.*, 1997). But while they do have the right scale, they frequently contain residual growth factors, undefined constituents, or nonquantified impurities. It is thus very difficult to conduct a completely controlled study using these biomaterials because they vary from lot to lot. This not only makes it difficult to conduct a well-controlled study but also would pose problems if such scaffolds were ever used to grow tissues for human therapies. Animal-derived biomaterials, for example, collagen gels, laminin, poly-glycosaminoglycans, and materials from basement membranes including Matrigel™, have been used as an alternative to synthetic scaffolds (Bissell *et al.*, 2002; Cukierman *et al.*, 2001, 2002; Kleinman *et al.*, 1986; Kubota *et al.*, 1981; Lee *et al.*, 1985; Oliver *et al.*, 1987; Schmeichel and Bissell, 2003; Weaver *et al.*, 1995; Zhau *et al.*, 1997). Although researchers are well aware of their limitations, it is one of the few limited choices. Thus, it not only makes it difficult to conduct reproducible studies but also would pose problems if these scaffolds will be used to grow tissues for human therapies (Tables I and II).

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Table I A Variety of Tissue Cells Cultured on the Designer Self-Assembling Peptide Nanofiber Scaffolds

Chicken embryo fibroblast	Bovine calf and adult chondrocytes
Mouse fibroblast	Bovine endothelial cells
Mouse embryonic stem cells	Mouse adult neural stem cells
Mouse cerebellum granule cells	Mouse and rat hippocampal cells
Mouse mesenchymal stem cells	Mouse cardiac myocytes
Rat adult liver progenitor cells	Rat liver hepatocytes
Rat pheochromocytoma	Rat cardiac myocytes
Rat neural stem cells	Rat hippocampal neural tissue slice
Bovine osteoblasts	Bovine endothelium cells
Chinese hamster ovary	Hamster pancreas cells
Horse bone marrow	Rat keratinocytes
Human cervical carcinoma	Human osteosarcoma
Human hepato-cellular carcinoma	Human neuroblastoma
Human embryonic Kidney	Human Hodgkin's lymphoma
Human epidermal keratinocytes	Human foreskin fibroblast
Human neural stem cells	human aortic endothelial cells

Note: These cells include stable cell lines, primary isolated cells from animals, progenitor, and adult stem cells.

Table II Animals that Have Been Exposed to PeptideNanofiber Scaffolds

Mice
Rats
Hamsters
Rabbits
Goats
Monkeys
Pigs
Horses

Note: These animals were tested in various academic laboratories and commercial testing laboratories as well as biomaterials and medical device companies around the world.

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An ideal 3-D culture system that can be fabricated from a synthetic biological material with defined constituents of truly biological origin is thus required. Thus the molecular designer self-assembling peptide nanofiber scaffolds may be a promising alternative. We directly compared the Matrigel with the designer self-assembling peptide nanofiber scaffold (Fig. 2). They have similar nanoscales and similar porosity except Matrigel, which seems to have many particles, perhaps proteins that are contained in the Matrigel. On the other hand, the peptide nanofibers are very smooth, suggesting their purity and homogeneous structure (Fig. 2).

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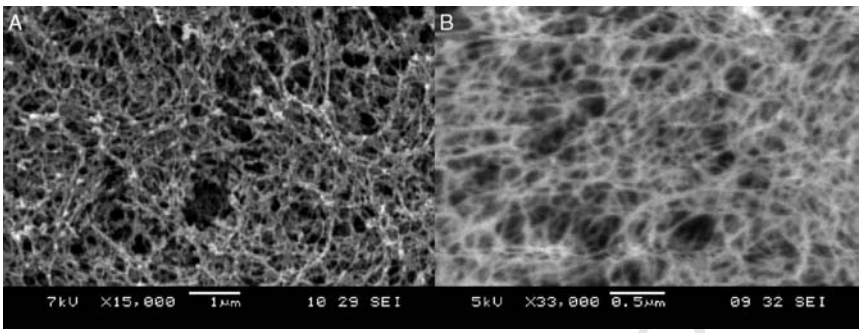

Fig. 2 SEM images of Matrigel and designed self-assembling peptide nanofiber scaffold. (A) Matrigel 0.5-μm scale bar (40,000X magnifications). (B) RADA16-I (33,000X magnifications) 0.5-μm scale bar. These peptides all form nanofiber scaffolds with nanopores (average 5–200 nm). It is worth noting that the nanopores may allow small molecular drugs (1–2 nm) and proteins (2–10 nm) to diffuse in the scaffolds slowly. This is in sharp contrast to many other biopolymer microfiber materials where the pores are also microns that drugs and proteins diffuse rather quickly. (Image courtesy of Fabrizio Gelain).

IV. THE IDEAL BIOLOGICAL MATERIALS

Although there are a number of criteria to fabricate biological scaffolds, the ideal 3-D biological scaffolds should meet several important criteria: (1) the building blocks should be derived from true biological sources; (2) 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) afford economically scalable and reproducible material production, purification, and processing; (7) be readily transportable; (8) be chemically compatible with aqueous solutions and physiological conditions; (9) elicit no or little immune responses and inflammation if used in human therapies; and (10) integrate with other materials and tissues in the body.

V. DISCOVERY OF SELF-ASSEMBLING PEPTIDE SCAFFOLDS

The self-assembling peptide scaffold belongs to a class of biologically inspired materials. The first member, EAK16-II (AEAEAKAKAEAEAKAK), of the family was discovered from a segment in a yeast protein, Zuotin (Zhang *et al.*, 1993). The scaffolds consist of alternating amino acids that contain 50% charged residues (Caplan *et al.*, 2002; Gelain *et al.*, 2006; Holmes *et al.*, 2000; Horii *et al.*, 2007; Kisiday *et al.*, 2002; Zhang *et al.*, 1993, 1994, 1995). These peptides are characterized by their periodic repeats of alternating ionic

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hydrophilic and hydrophobic amino acids with a typical β-sheet structure. Thus, these β-sheet peptides have distinct polar and nonpolar surfaces. The self-assembly event creating the peptide scaffold takes place under physiological conditions. They are like gel-sponge in aqueous solution and readily transportable to different environments. Individual fibers are $\sim$10 nm in diameter. A number of additional self-assembling peptides including RADA16-I (AcN-RADARADARADARADA-CNH2) and RADA16-II (AcN-RARADADAR-ARADADA-CNH2), in which arginine and aspartate residues substitute lysine and glutamate, have been designed and characterized for salt-facilitated nanofiber scaffold formation. The alanines form overlap hydrophobic interactions in water, a structure that is found in silk fibroin from silkworms and spiders. 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 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 nanofiber retains extremely high hydration, $>$99% in water (5–10 mg/ml, w/v) (Fig. 3).

The peptide synthesis method uses conventional mature solid phase or solution peptide synthesis chemistry. Depending on the length of the motifs, highly pure peptides can be produced at a reasonable cost. Since the cost of peptide synthesis has decreased steadily in the past few years, it has become affordable for most people.

Many self-assembling peptides that form scaffolds have been reported and the numbers are still expanding (Zhang, 2002; Zhang and Altman, 1999). The formation of the scaffold and its mechanical properties are influenced by several factors, one of which is the level of hydrophobicity (Caplan *et al.*, 2002; Marini *et al.*, 2002]. 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), can significantly influence the mechanical properties of the scaffolds and the speed of their self-assembly. The higher the content of hydrophobicity, the easier it is for scaffold formation and the better for their mechanical properties (Caplan *et al.*, 2002; Kisiday *et al.*, 2002; Marini *et al.*, 2002).

VI. SELF-ASSEMBLING PEPTIDE NANOFIBER SCAFFOLDS

A single molecule of the ionic self-complementary peptide RADA16-I is shown in Figs. 3 and 4. Millions of peptide molecules self-assembled into individual nanofibers that further form the nanofiber scaffold (Fig. 3). The nanopores range from a few nanometers to a few hundred nanometers; the scales are similar in size as most biomolecules, so that these molecules or

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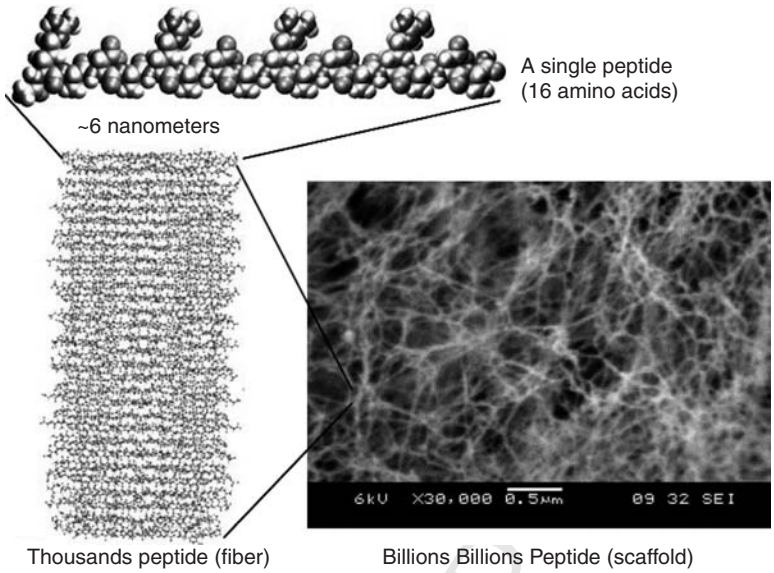

Fig. 3 Self-assembling peptide RADA16-I nanofiber scaffold hydrogel. (A) Amino acid sequence of RADA16-I, molecular model of a single RADA16-I nanofiber, the dimensions are ~6-nm long, 1.3-nm wide, and 0.8-nm thick; (B) tens and hundred thousands of individual peptides self-assemble into a nanofiber; and (C) SEM images of RADA16-I nanofiber scaffold. Note the scale bar, 0.5 μm or 500 nm. (SEM image courtesy of Fabrizio Gelain). (See Color Insert.)

drugs may not only defuse slowly but also establish a molecular gradient in the scaffolds. Figure 4 shows the individual nanofibers ranging from a few hundred nanometers to a few microns. Peptide samples in aqueous solution, using environmental AFM examination, showed similar nanofiber results, suggesting that the nanofiber formation is independent of the drying process. It is interesting to observe that at high resolution the nanofibers appeared to have distinct layers, especially in some segments (Fig. 4D). The difference in height was about 1.3–1.5 nm, the similar dimension as a single thickness of a peptide. Figure 4E–H shows the peptide scaffold hydrogel at various concentrations, 0.6–3 mM (1–5 mg/ml, w/v, or 99.5–99.9% water content) (Yokoi *et al.*, 2005). The scaffold hydrogel is completely transparent, which is a very important requirement for accurate image collections for uses in 3-D tissue cell cultures.

VII. DYNAMIC REASSEMBLY OF SELF-ASSEMBLING PEPTIDES

The self-assembling process is reversible and dynamic (Fig. 5). Since these peptides are short and simple, numerous individual peptides can be readily self-organized through weak interactions including hydrogen bonds, ionic

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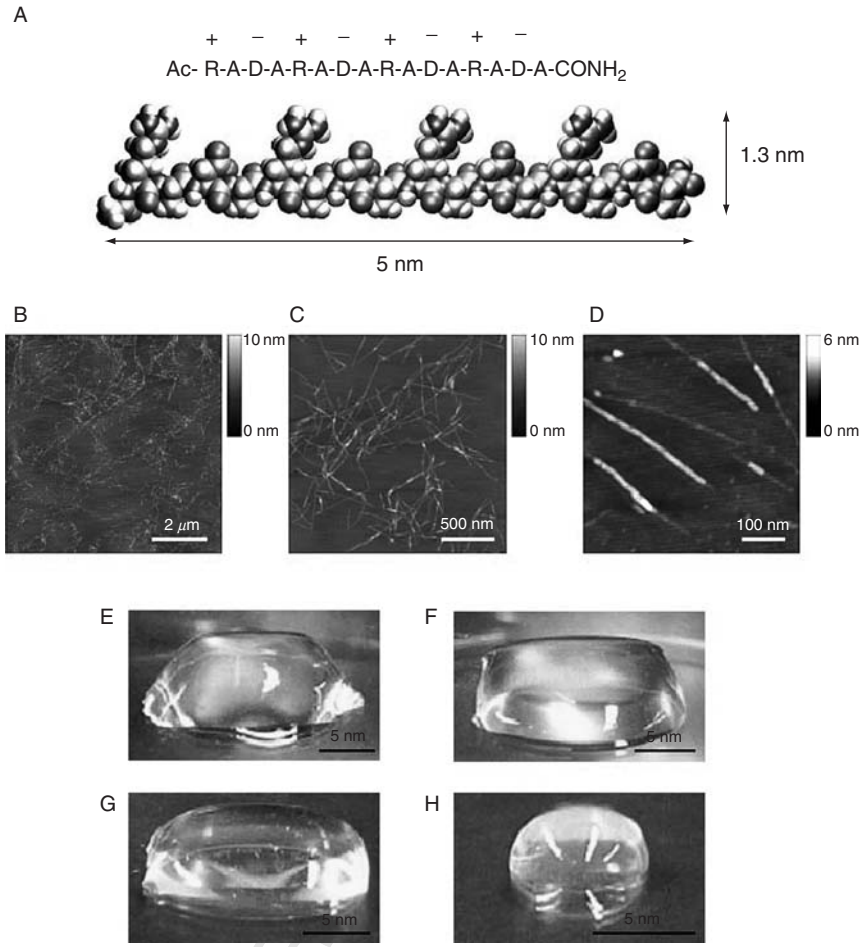

Fig. 4 Peptide RADA16-I. (A) Amino acid sequence and molecular model of RADA16-I, the dimensions are ~5-nm long, 1.3-nm wide, and 0.8-nm thick; (B) AFM images of RADA16-I nanofiber scaffold, 8 μm × 8 μm, (C) 2 μm × 2 μm (D) 0.5 μm × 0.5 μm. Note the different height of the nanofiber, ~1.3 nm, in D suggesting 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, Tris. HCl), (G) 0.1 wt% (pH 7.5, PBS) before sonication, (H) reassembled RADA16-I hydrogel after 4 times of sonication, respectively (images courtesy of Hidenori Yokoi). (See Color Insert.)

bonds, hydrophobic and van der Waals interactions as well as water-mediated hydrogen bond formations. These nanofibers can be broken mechanically with sonication (Yokoi *et al.*, 2005). However, they can undergo dynamic reassembly repeatedly, similar as the material self-healing process (Fig. 5). Since the driving energy of the assembly in water is not only through hydrophobic van der Waals interactions but also through the arrays of ionic interactions as well as the peptide backbone hydrogen bonds, this

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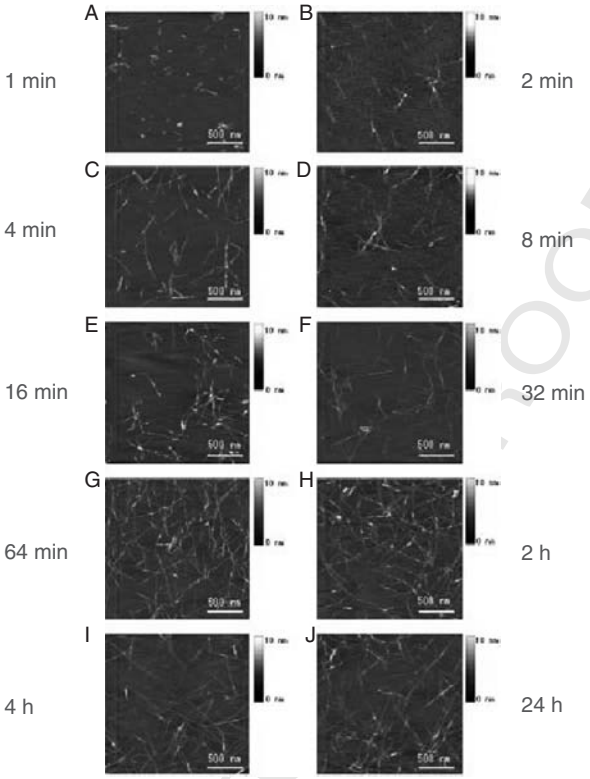

Fig. 5 AFM images of RADA16-I nanofiber at various time points after sonication. The observations were made using AFM immediately after sample preparation. (A) 1 min after sonication; (B) 2 min; (C) 4 min; (D) 8 min; (E) 16 min; (F) 32 min; (G) 64 min; (H) 2 h; (I) 4 h; and (J) 24 h. Note the elongation and reassembly of the peptide nanofibers over time. By ~1–2 h, these self-assembling peptide nanofibers have nearly fully reassembled (images courtesy of Hidenori Yokoi). (See Color Insert.)

phenomenon can be further exploited for production and fabrication of many self-assembling peptide materials.

Unlike processed polymer microfibers in which the fragments of polymers cannot readily undergo reassembly without addition of catalysts or through material processing, the supramolecular self-assembly and reassembly event is likely to be widespread in many unrelated fibrous biological materials where numerous weak interactions are involved. Self-assembly and reassembly are very important properties for fabricating novel materials, and it is necessary to fully understand their detailed process in order to design better biological materials. Au10

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AFM images revealed that the nanofibers range from several hundred nanometers to a few microns in length before sonication. After sonication, the fragments were broken into ~20–100 nm. The kinetics of the nanofiber reassembly is followed closely at 1, 2, 4, 8, 16, 32, and 64 min as well as at 2, 4, and 24 h (Fig. 5). The nanofiber length reassembly is a function of time: by 2 h, the peptide nanofibers have essentially reassembled to their original length. This remarkable and rapid reassembly is interesting because there may be a little nucleation for regrowth of the nanofiber from the addition of monomers that could only be produced during sonication. It is plausible that a large population of the sonicated nanofiber fragments contains many overlap cohesive ends due to an undisrupted alanine hydrophobic side that may quickly find each other (Fig. 4D). The situation is analogous and commonly found in sonicated and enzymatic digested DNA fragments.

VIII. KINETICS OF NANOFIBER REASSEMBLY AND A PLAUSIBLE REASSEMBLY PROCESS

The reassembly kinetics is a function of time. Perhaps, similar to DNA reassembly, the reassembly largely depends on the concentrations of the short complementary fragments. In this case, the fragments are the sonicated peptide nanofibers with possible presence of sonicated monomers.

In order to understand the dynamic reassembly, we proposed a plausible sliding diffusion molecular model to interpret these observations of reassembly of the self-assembling RADA16-I peptides (Fig. 6). Unlike the left-handed helical structures observed in KFE8 (Marini *et al.*, 2002), a different self-assembling peptide, no helical structures were observed for RADA16-I using AFM and TEM (Gelain *et al.*, 2006; Holmes *et al.*, 2000).

For molecular modeling clarity, these RADA16-I β-sheets are presented as nontwisted strands. It is known that these peptides form stable β-sheet structure in water; thus they not only form the intermolecular hydrogen bonding on the peptide backbones but also have two distinctive sides, one hydrophobic with an array of overlapping alanines (Fig. 6, green color sandwiched inside), similar to that found in silk fibroin or spider silk assemblies (Pauling, 1961) and the other with negatively charged (−) aspartic acids, represented as red, and positively charged (+) arginines, represented as blue.

The alanines form packed hydrophobic interactions in water; during sonication the hydrophobic interaction could be disrupted mechanically. However, these hydrophobic cohesive ends could find each other quickly in water since the exposure of hydrophobic alanine arrays to water is energetically unfavorable. Since the hydrophobic alanines interaction is nonspecific, they can slide diffuse along the nanofiber, similar to trains on the train tracks. The same

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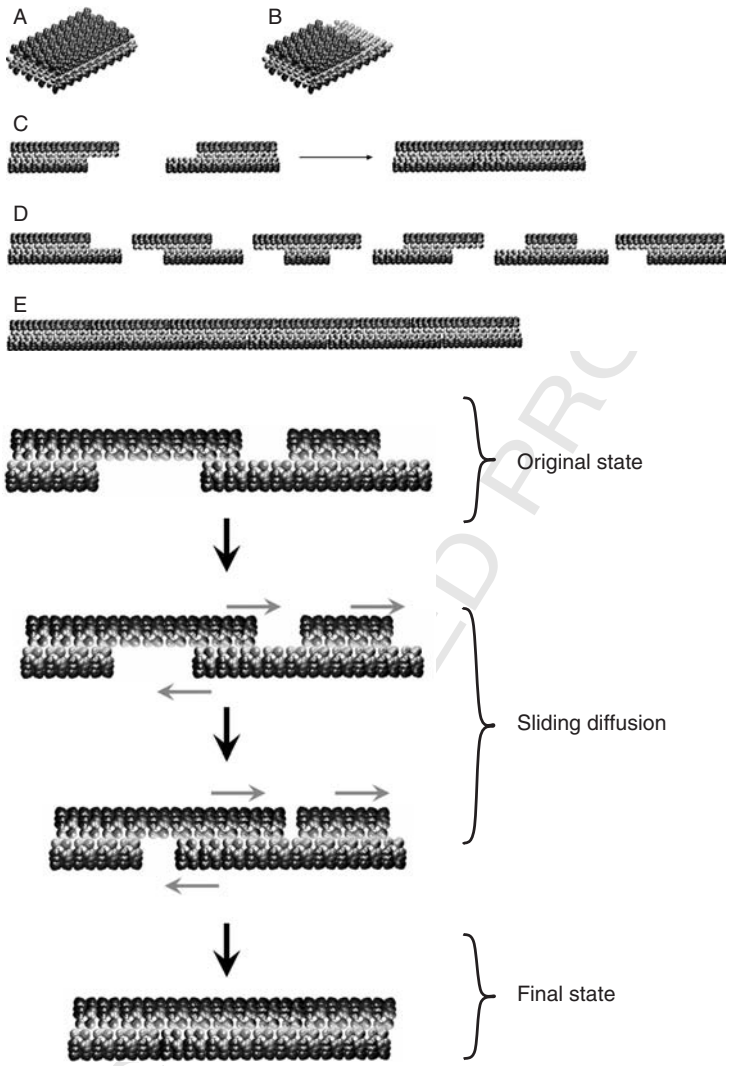

Fig. 6 A proposed molecular sliding diffusion model for dynamic reassembly of self-assembling RADA16-I peptides. When the peptides form stable β-sheets in water, they form intermolecular hydrogen bonds along the peptide backbones. The β-sheets have two distinctive sides, one hydrophobic with an array of alanines and the other with negatively charged aspartic acids and positively charged arginines. 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 silkworms 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: (A) blunt ends; (B) semiprotruding ends. (C) These fragments with protruding ends could reassemble readily through hydrophobic interactions. (D) The fragments with semiprotruding

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sliding diffusion phenomenon was also observed in nucleic acids where polyA and polyU form complementary base pairings that can slide diffuse along the chains (Felsenfeld *et al.*, 1957; Rich and Davies, 1956). If however, the bases are heterogeneous, containing G, A, T, and C, the bases cannot undergo sliding diffusion. Likewise, if the hydrophobic side of the peptides does not always contain alanine, such as valine and isoleucine, it would become more difficult for sliding diffusion to occur because of structure constraint.

On the charged side, both positive and negative charges are packed together through intermolecular ionic interactions in a checkerboard manner (looking from the top). Likewise, collectively complementary + and − ionic interactions may also facilitate the reassembly. Similar to restriction-digested DNA fragments, these nanofiber fragments could form various assemblies: blunt, semiprotruding, and protruding ends. The fragments with semiprotruding and various protruding ends as well as those with blunt ends can reassemble readily through hydrophobic and ionic interactions.

IX. SELF-ASSEMBLING PEPTIDES NANOFIBER SCAFFOLD 3-D CELL CULTURE

The importance of nanoscale becomes obvious in 3-D cell culture. It is clearly visible in the SEM images that the cells are embedded in the self-assembling peptide nanofiber biological scaffolds in the truly 3-D culture (Fig. 7). Here, the cells and cell clusters intimately interact with the extracellular matrix where cells make on their own over time during cell growth and differentiation. Since the scaffolds are made of mostly water, ~99% water

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and various protruding ends. (E) These fragments can reassemble readily. A proposed molecular sliding diffusion model for dynamic reassembly of self-assembling a single peptide nanofiber consisting of thousands of individual 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. 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 silkworms and spiders. On the charged sides, both positive and negative charges are packed together through intermolecular ionic interactions in a checkerboard-like manner. When the fragments of nanofiber first meet, the hydrophobic sides may not fit perfectly but with gaps. However, the nonspecific hydrophobic interactions permit the nanofiber to slide diffusion along the fiber in either direction, which minimizes the exposure of hydrophobic alanines and eventually fills the gaps. The sliding diffusion phenomenon was also proposed for nucleic acids of polyA and polyU in 1956 (Felsenfeld *et al.*, 1957; Rich and Davies, 1956). For clarity, these β-sheets are not presented as twisted strands. Color code: green, alanines; red, negatively charged aspartic acids; blue, positively charged arginines (images courtesy of Hidenori Yokoi). (See Color Insert.)

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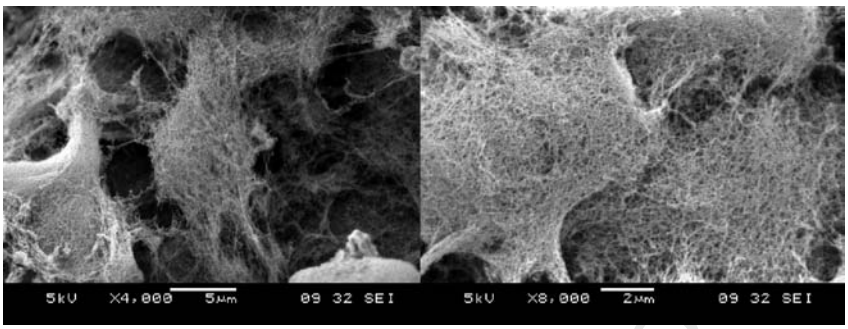

Fig. 7 Clusters of cells are fully embedded in the self-assembling peptide nanofiber scaffold. The scales of the nanofibers are similar to those of the extracellular matrices. Furthermore, the factors secreted from cells do not diffuse away quickly; thus a local concentration gradient could likely form, which is an absolute requisite for tissue development. Such 3-D cell clusters are nearly impossible to form on the 2-D petri dish and other 2-D culture systems. Likewise, several biopolymer microfibers commonly used in tissue engineering and regenerative medicine do not show such intimate cell–matrix interactions. Although there are similar cell–matrix interactions in Matrigel, it can never be used for human therapies. These cell clusters may be eventually form 3-D tissue over time under the appropriate conditions. There are few tissue examples that are shown in Fig. 5 (image courtesy of Fabrizio Gelain).

with 1% peptide, cells can migrate freely without hindrance, just as fish swim freely in a seaweed forest.

These new self-assembling peptide nanofiber biological scaffolds have become increasingly important not only in studying 3-D spatial behaviors of cells but also in developing approaches for a wide range of innovative medical technologies, including regenerative medicine (Fig. 8). One example is the use of peptide scaffolds to support neurite growth and differentiation, neural stem cell (NSC) differentiation, cardiac myocytes, and bone and cartilage cell cultures. The peptide scaffolds from RADA16-I and RADA16-II formed nanofiber scaffold in physiological solutions that stimulated extensive rat neurite outgrowth and active synapses formation on the peptide scaffold (Holmes *et al.*, 2000).

X. DESIGNER PEPTIDES SCAFFOLD 3-D CELL CULTURES

In a recent work, we directly and systematically compared NSC adhesion and differentiation on self-assembling RADA16-I scaffolds with other nature-based substrates including laminin, Collagen I, fibronectin, and some of the most commonly used synthetic biomaterials in tissue engineering such as poly-(DL-lactic acid), poly-(lactide-co-glycolide acid), and poly-(capro-lactone acid)

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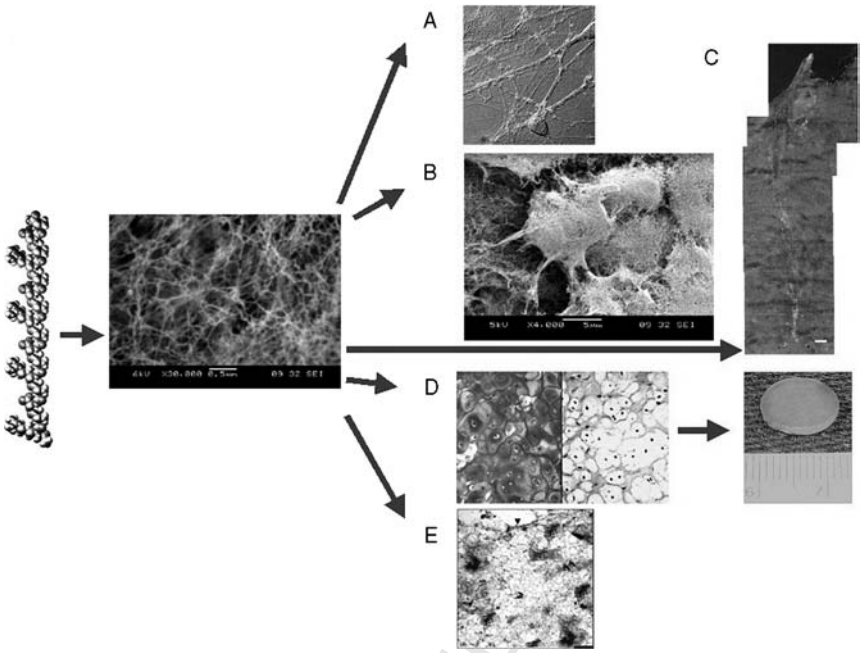

Fig. 8 From designer peptide to scaffold to tissues. (A) Active synapses on the peptide surface. Primary rat hippocampal neurons form active synapses on peptide scaffolds. The confocal images show bright discrete green dot labeling indicative of synaptically active membranes after incubation of neurons 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 NSC embedded in 3-D scaffold (image courtesy of Fabrizio Gelain). (C) Brain damage repair in hamster. The peptide scaffold was injected into the optical nerve area of brain that was first severed with a knife. The cut was sealed by the migrating 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 showing abundant GAG production (left panel) and antibody to type II collagen demonstrating 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 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). (See Color Insert.)

(Gelain *et al.*, 2007). While nature-derived substrates showed the best performances, RADA16-I scaffold stimulated NSC differentiation and survival to a similar degree as did other synthetic biomaterials.

Although self-assembling peptides are promising scaffolds, they show no specific cell interaction because their sequences are not naturally found in

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living systems. The next logical step is to directly couple biologically active and functional peptide motifs with a wealth of literature; accordingly the second generation of designer scaffolds will significantly enhance their interactions with cells and tissues.

The simplest way to incorporate the functional motifs is to directly synthesize them by extending the motifs onto the self-assembling peptides themselves (Fig. 9). The functional motifs are on the C-termini since peptide synthesis start from C-termini to avoid deletion during synthesis. Usually, a spacer comprising 2 glycines 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 in the same scaffold. Upon exposure to solution at neutral pH, the functionalized sequences self-assemble, leaving the added motifs on both sides of each nanofiber (Fig. 9). Nanofibers take part to the overall scaffold, thus giving microenvironments functionalized with specific biological stimuli (Fig. 9).

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

We have produced different designer peptides from a variety of functional motifs with different lengths (Gelain *et al.*, 2006; Horii *et al.*, 2007). We showed that the addition of motifs to the self-assembling peptide RADA16-I did not inhibit self-assembling properties and nanofiber formations through mixing the modified peptides with the original RADA16-I. Although their nanofiber structures appear to be indistinguishable from the RADA16-I scaffold, the appended functional motifs significantly influenced cell behaviors.

Using the designer self-assembling peptide nanofiber system, every ingredient of the scaffold can be defined and, furthermore, can be combined with multiple 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, whereas cells reside in a 3-D environment where the extracellular matrix receptors on the cell membranes can bind to the functional ligands appended to the peptide scaffolds. It is likely that higher tissue architectures with multiple cell types, rather than monolayers, could be constructed using these designer 3-D self-assembling peptide nanofiber scaffolds (A. Schneider *et al.*, unpublished results).

Even if only a fraction of functionalized motifs on the 3-D scaffold are available for cell receptor binding, cells may likely receive more external stimuli than when in contact with coated 2-D petri dishes or RGD-coated (or other motifs) polymer microfibers, which is substantially larger than the cell

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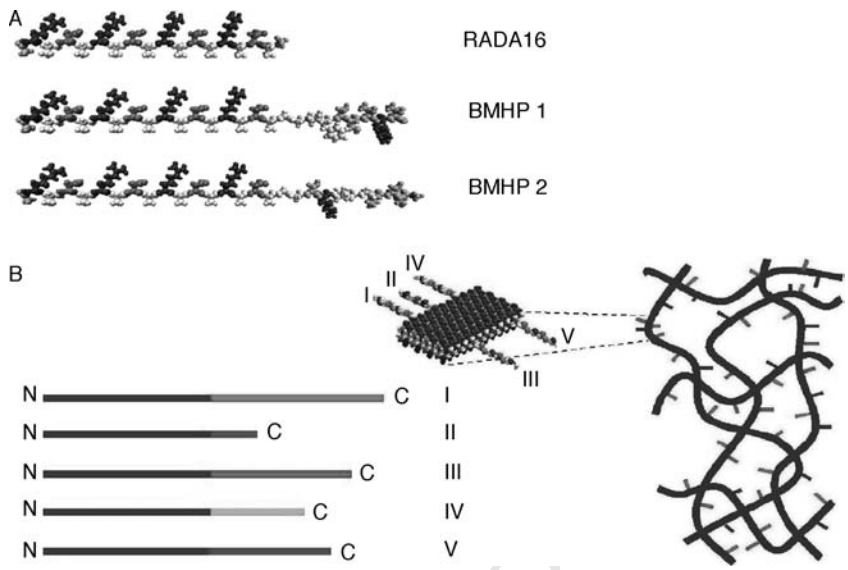

Fig. 9 Molecular and schematic models of the designer peptides and of the scaffolds. (A) Molecular models of RADA16, RADA16-Bone Marrow Homing Peptide 1 (BMHP1), and RADA16-Bone Marrow Homing Peptide 2 (BMHP2). RADA16 is an alternating16-residue peptide with basic arginine (blue), hydrophobic alanine (white), and aspartic acid (red). These peptides self-assemble once exposed to physiological pH solutions or salt. The alanines of the RADA16 providing hydrophobic interaction are on one side of the peptide, and the arginines and aspartates form complementary ionic bonds on the other. The BMHP1 and BMHP2 motifs were directly extended from RADA16 with two glycine spacers and are composed of a lysine (blue), serine and threonine (green), and different hydrophobic (white) residues. Neutral polar residues are drawn in green. (B) Schematic models of several different functional motifs (different colored bars) could be extended from RADA16 (blue bars) in order to design different peptides (I, II, III, IV, and V). They can be combined in different ratios. A schematic model of a self-assembling nanofiber scaffold with combinatorial motifs carrying different biological functions is shown. (See Color Insert.)

surface receptors and, in most cases, larger than the cell themselves. There cells are not in real 3-D; rather, they are in 2-D wrapping around the micropolymers 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 sides that are exposed directly to 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 nano porous microenvironment.

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In our search for additional functional motifs, we found that a class of bone marrow homing peptides (BMHP) (Gelain, *et al.*, 2006, 2007) is one of the most promising active motifs for stimulating adult mouse NSC adhesion and differentiation. This observation suggests a new class of designer self-assembling peptides for 3-D cell biology studies.

XI. DESIGNER PEPTIDE SCAFFOLDS FOR BONE CELLS AND 3-D MIGRATION

The designer self-assembling peptide nanofiber scaffolds have been shown to be an excellent biological material for 3-D cell cultures and capable of stimulating cell migration into the scaffold as well as repairing tissue defects in animals. We developed several peptide nanofiber scaffolds, designed specifically for osteoblasts (Horii *et al.*, 2007). 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). The new peptide scaffolds were made by mixing the pure RADA16-I and designer peptide solutions, and the molecular integration of the mixed nanofiber scaffolds was examined using AFM. Compared to pure RADA16-I scaffold, it was 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, 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 unequivocally demonstrated that the designer PRG peptide scaffold stimulated cell migration into the 3-D scaffold (Fig. 10) (Horii *et al.*, 2007). Without the modified motif, cells did not migrate in 3-D.

XII. WHY DESIGNER SELF-ASSEMBLING PEPTIDE SCAFFOLDS?

One may ask why one should choose designer self-assembling peptide scaffolds while there are a large number of biomaterials on the market. The advantages of using the designer peptide nanofiber scaffolds are severalfold.

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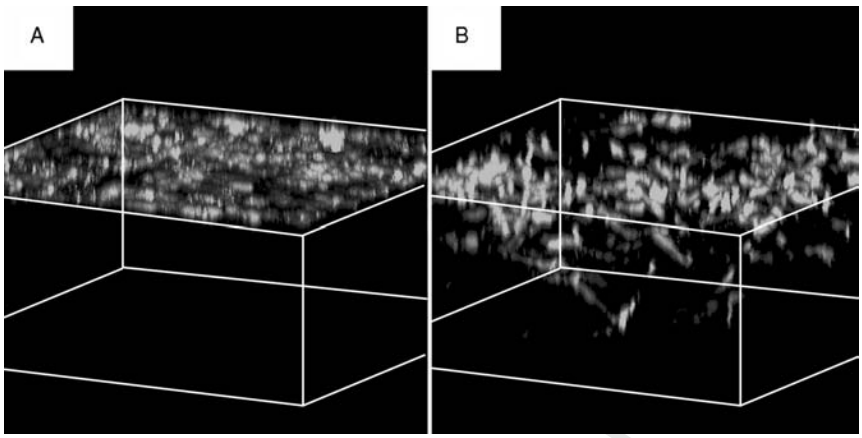

Fig. 10 Reconstructed image of 3-D confocal microscopy image of culturing on the different scaffolds consisting of different mix ratio of RADA16-I 1% (w/v) and PRG 1% (w/v) using calcein-AM staining. (A) PRG 10% and (B) PRG 70% of designer self-assembling peptide nanofiber scaffolds. The confocal images are horizontal view. There is a drastic cell migration into the scaffold with higher concentration of PRG motif (images courtesy Akihiro Horii). (See Color Insert.)

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(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 contains pure components and every ingredient is completely defined. (3) Because these designer peptide scaffolds are pure with known motifs, they 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 the change of ionic strength at physiological conditions without temperature influence. This is again unlike collagen gels, for which gelation is through the change of temperature, which can sometimes induce unknown biological processes 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 bio-reabsorbable scaffolds.

Although we have not studied cancer and tumor cells in our laboratory, others have carried out experiments for such studies. J. K. Park's group of

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Korea and colleagues reported using the peptide scaffold to study human hepatocellular carcinoma cells (Kim *et al.*, 2007). Ingemar Ernberg's laboratory in Karolinska Institute also used the peptide scaffold to study Human Hodgkin's lymphoma [personal communication, 2007]. Lisa Spiro, then in Robert Weinberg's laboratory at the Whitehead Institute, also used the peptide scaffold to study cancer cells [personal communication]. So the time has come to study tumor and cancer cells using the designer self-assembling peptide nanofiber scaffold 3-D cell culture systems. Au11

XIII. BEYOND 3-D CELL CULTURES

Researchers in neuroscience have a strong desire to study neural cell behaviors in 3-D and to fully understand their connections and information transmission (Edelman and Keefer, 2005). Beyond 3-D cell culture, since the building blocks of this class of designer peptide scaffolds are natural L-amino acids, the RADA16 has been shown not to elicit noticeable immune response nor inflammatory reactions in animals (Davis *et al.*, 2005, 2006; Ellis-Behnke *et al.*, 2006; Zhang *et al.*, 2005). The degraded product**s, a**mino acids, can be reused by the body and may also be useful as a bio-reabsorbable scaffold for neural repair and neuroengineering to alleviate and to treat a number of neurotrauma (Ellis-Behnke *et al.*, 2006) and neurodegeneration diseases.

In a recent work led by Richard Lee, mouse embryonic stem cells were suspended in RADA16-II peptide scaffold solutions and injected in the myocardium of 10-week-old mice (Davis *et al.*, 2005). 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 (Davis *et al.*, 2006). 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.

Ellis-Behnke and colleagues showed that self-assembling peptide material is a promising scaffold for neural regeneration medicine (Ellis-Behnke *et al.*, 2006). *In vivo* application to brain wounds was carried out using postnatal

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day-2 Syrian hamster pups. The optic tract within the superior colliculus (SC) was completely severed with a deep knife wound, extending at least 1mm below the surface. At surgery, 10 animals were treated by injection of 10–30 μl of 1% RADA16/99% water, w/v into the wound). Control animals with the same brain lesion included 3 with isotonic saline injection (10 μl), 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 at 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 important, functional tests proved a significant restoration of visual function in all peptide scaffold-treated animals. Au12

During the brain surgery experiments, Ellis-Behnke and colleagues found that the peptide nanofiber scaffold hydrogel could also stop bleeding in less than 15 s (Ellis-Behnke *et al.*, 2007). This is unlikely to be the conventional blood clogging mechanism because it takes place so rapidly. The molecular mechanism of speedily stopping bleeding remains to be uncovered. It is plausible that the nanofibers self-assembled at the site quickly self-assembled into a dense mesh nanofiber network sponge that instantly blocked the rushing of the liquid. It may be perhaps nanomechanics rather than biochemistry.

The development of new biological materials, particularly those biologically inspired nanoscale scaffolds mimicking *in vivo* environment that serve as permissive substrates for cell growth, differentiation, and biological function, is an actively pursued area that, in turn, could significantly advance regenerative medicine. These materials will be useful not only to further our understanding of cell biology in 3-D environment but also for advancing medical technology, regenerative biology, and medicine.

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