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Designer Self-Assembling Peptide Materials for Diverse Applications

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Summary: Macromolecuar chemistry and polymer science have had an enormous impact in many areas of science, engineering, medicine and our daily life. It has not only changed our way of life forever, but also continuously to improve our living standard. Macromolecuar chemistry now also encompasses bioengineering, biomimetics, designer biological materials and nanobiotechnology. Here we summarize a few classes of short peptides that we discovered and invented with broad applications including 3D tissue cell culture, reparative and regenerative medicine, tissue engineering, slow drug release, stabilization of membrane proteins for develop nanobiotechnology and molecular devices. Molecular design using short peptides as new materials will play increasingly important role in biomedical research, nanobiotechnology, clinical science and medicine.

Keywords: 3D cell cultures; ionic self-complementary peptides; lipid-like peptides; molecular self-assembly; stabilizing membrane proteins; tissue regeneration

Introduction

Since early macromolecuar chemistry and polymer science were studied, they immediately have had an enormous impact in many areas of science, engineering medicine and our civilization. It not only then stimulated and spurred many new industries, but it also produced numerous inexpensive products that have forever changed our way of life and continuously to improve our living standard. The Institute of Macromolecuar Chemistry in Prague, Czech Republic played a leading role in advancing macromolecuar chemistry even under extremely difficult cold war political conditions and with very limited resources. However, undeterred, the scientists at the Institute established numerous scientific collaborations with other European countries and United States. They shared the ideas, unpublished research results and exchanged research materials. These open sharing of scientific ideas and continuous collaborations eventually accelerated many discoveries and products that benefit the society. They set an excellent example for others to follow. Hope their examples and the trends continue elsewhere in the world. And that will make the world a better place.

Macromolecuar chemistry in the early era mostly concerned the design and synthesis of a wide range of organic polymers and organic and inorganic composites. As all areas of science, macromolecuar chemistry has evolved and now also encompasses bioengineering, biomimetics, designer biological materials and nanobiotechnology. These fields have been rapidly advancing in a breathless pace in recent years. Enormous amount funding has been poured into these endeavors; numerous polymer programs, centers, institutes and innovative technology start-ups have mushroomed worldwide and the trend

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is accelerating. Reently, the macromolecular chemistry has reinvented itself to include nano-materials, nanoscience and nanotechnology.

Our research is in the area of designer self-assembling peptide materials. It is in the general sense, belongs to the macromolecular chemistry. How we get into this type of research is an interesting story itself. We both worked in the Molecular Structure Laboratory of Alexander Rich at Massachusetts Institute of Technology in late 1980s and 1990s to study the biological functions of left-handed Z-DNA. Z-DNA is the rare and less stable form of DNA structure that involves in a number of important biological regulations through Z-DNA binding proteins. Thus, Rich's laboratory then actively and rigorously pursued and even now continuously pursues all aspects of biology aspects of left-handed Z-DNA. While working on yeast genetics and protein chemistry and trying to understand a left-handed Z-DNA structure in 1989, one of us identified a protein called Zuotin for its ability to bind to left-handed Z-DNA in the presence of 400-fold excess of sheared salmon sperm DNA that contains ubiquitous right-handed B-DNA and other random DNA structures.[1] Zuotin had an interesting repetitive 16-residue peptide sequence motif n-AEAEAKA-KAEAEAKAK-c (EAK16-II).[2] This peptide has been extensively developed to create a class of simple β-sheet peptides. These peptides are ionic self-complementary as a result of the presence of both positive and negative side chains on one side of the beta-sheet and hydrophobic side chains on the other (Figure 1). These peptides have two distinctive sides, one hydrophobic and the other hydrophilic. The hydrophobic side forms a double sheet inside of the fiber and hydrophilic side forms the outside of the nanofibers that interact with water molecules that they can form extremely high water content hydrogel, containing as high as 99.5% to 99.9% water (1 mg–5 mg peptide/ml water). At least three types of molecules can be made,

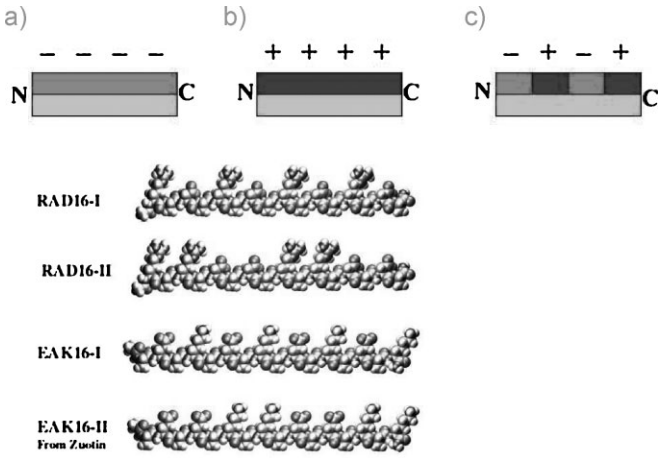

Figure 1.
A) The designer amphiphilic self-assembling peptides that form well-ordered nanofibers. These peptides have two distinctive sides, one hydrophobic and the other hydrophilic. The hydrophobic side forms a double sheet inside of the fiber and hydrophilic side forms the outside of the nanofibers that interact with water molecules that they can form extremely high water content hydrogel, containing as high as 99.9% water. At least three types of molecules can be made, with −, +, −/+ on the hydrophilic side. B) The individual self-assembling peptide molecules are 5 nm long. The first such peptide, EAK16-II, was discovered from a yeast protein, zuotin. This peptide inspired us to design a large class of self-assembling peptide construction motifs. Upon dissolve in water in the presence of slat, they spontaneously assemble into well-ordered nanofibers, further into scaffolds.

with −, +, −/+ on the hydrophilic side (Figure 1).[3]

This discovery stimulated new thinking that short peptides cannot only be designed as a variety of stable structures from bottom up with exquisitely fine-tuning to build novel materials one amino acid at a time, but it also laid the foundations for fabrication of a wide spectrum of new materials and devices.

For the proceedings of the 50-year celebration of Institute of Macromolecular Chemistry in Prague, we only focus our research highlights of designer peptides and their applications since 1993. Since this field is moving rapidly, it is impossible to cover the entire field with limited space. Thus, interested readers should consult other original articles and reviews in the literature to have a comprehensive view.

Discovery of the First Self-Assembling Peptide EAK16 in Yeast

In science, many interesting discoveries are made serendipitously; it is often referred as making unauthorized scientific discoveries. While one works in area but accidentally discovers something new in completely unrelated areas. The discovery of self-assembling peptides is another such example. However, after making the discovery, one has a choice either pursuit it or ignore it. We decided to pursue it. This pursuit has led a very stimulating and exciting scientific journey.

This serendipitous discovery of such an ionic self-complementary peptide inspired us to design many more members of this class of peptides, which self-assemble into 3-dimensional (3D) nanofiber scaffolds. These nanofiber scaffolds have been used in 3D cell tissue cultures[4–7] and other applications. Among this class of self-assembling peptides, 4 ionic self-complementary peptides (Figure 1) RDA16-I, RAD16-II, EAK-I and EAK16-II (the segment from yeast zuotin) are shown. They form stable β-sheet structures in water and undergo spontaneous assembly to form nanofiber scaffolds. The nanofiber scaffolds hold large volumes of water since water molecules can be organized to form clusters through surface tension and the nanofibers divide water clusters into compartments.[2,8] Tissue cells can be embedded in a 3D nanofiber scaffold[5–7] in which they can establish molecular gradients that often mimic the *in vivo* environment. Other related self-assembling peptide systems have also been designed, which range from 'molecular switch' peptides that undergo marked conformational changes[9,10] to 'molecular carpet' peptides for surface engineering[11] to peptide nanotubes and nanovesicles,[12–18] all of which were inspired from the Z-DNA-binding zuotin discovery.

The Property of the Self-Assembling Peptide Systems

The designer peptide scaffolds consist of alternating amino acids that contain 50% charged residues.[2–7,19–24] These peptides are characterized by their periodic repeats of alternating ionic hydrophilic and hydrophobic amino acids with a typical β-sheet structure. Thus, these β-sheet peptides have distinct polar and non-polar surfaces (Figure 1). The self-assembly event creating the peptide scaffold takes place under physiological conditions of neutral pH and milli-molar salt concentration. They are like gel-sponge in aqueous solution and readily transportable to different environments. Individual fibers are ~10 nanometers in diameter. A number of additional designer self-assembling peptides including RADA16-I (AcN-RADARADARADARADA-CNH_2) and RADA16-II (AcN-RARADADARARADADA-CNH_2), in which arginine and aspartate residues replace lysine and glutamate have been designed. The alanines form overlapping 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. 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 nanofibers retains extremely high hydration, >99% in water (1–10 mg/ml, w/v) (Figure 2).

The peptide synthesis method uses conventional mature solid phase or solution peptide synthesis chemistry. Depending on the length of the motifs, high purity of peptides can be produced at a reasonable cost. Since cost of the peptide synthesis has steadily decreased in recent years, it has become more and more affordable.

Many self-assembling peptides that form scaffolds have been reported and the numbers are still expanding.[3,23] The formation of the scaffold and its mechanical properties are influenced by several factors, one of which is the level of hydrophobicity.[20,24] 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.[20,22,24]

Self-Assembling Peptide Nanofiber Scaffolds

A hierarchical scaffold self-organization starting from a single molecule of the ionic self-complementary peptide RADA16-I is shown in Figure 2. Millions and billions of peptide molecules undergo self-assembly into individual nanofibers that further self-organize into the nanofiber scaffold (Figure 2). 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 drugs may not only defuse slowly but also may establish a molecular gradient in the scaffolds.

The AFM image shows the individual nanofibers ranging from a few hundred nanometers to a few microns. Peptide samples in aqueous solution using environmental AFM examination showed the similar nanofiber results suggesting the nanofiber formation is independent of drying process.

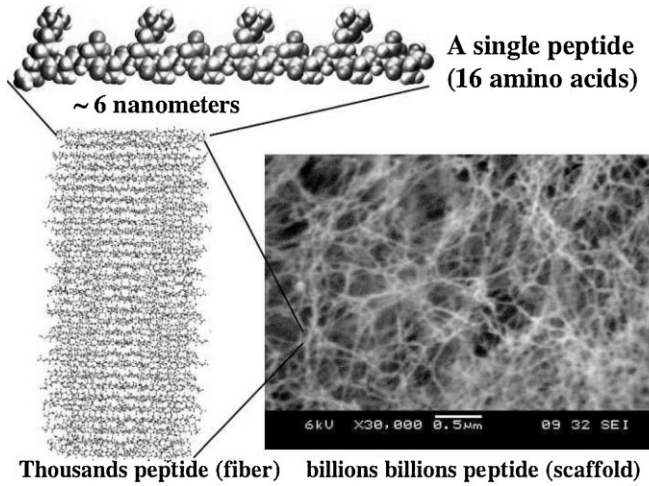

Figure 2.
A) Self-assembling peptide RADA16-I nanofiber scaffold hydrogel. Amino acid sequence of RADA16-I, molecular model of a single RADA16-I nanofiber, the dimensions are ~6 nm long, 1.3 nm wide and 0.8 nm thick; tens and hundred thousands of individual peptides self-assemble into a nanofiber. B) SEM images of RADA16-I nanofiber scaffold. Note the scale bar, 0.5 μm or 500 nm (SEM image courtesy of Fabrizio Gelain).

It is interesting to observe that at high resolution, the nanofibers appeared to have distinct layers, especially in some segments (Figure 3). The difference in height is about 1.3–1.5 nm, the similar dimension as a single thickness of a peptide. Figure 3 e-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).[8] The scaffold hydrogel is completely transparent, which is a very important requirement for accurate image collections for uses in 3-D tissue cell cultures.

Dynamic Reassembly of Self-Assembling Peptides

The self-assembling process is reversible and dynamic (Figure 4) since these peptides are short and simple, numerous individual peptides can be readily self-organized

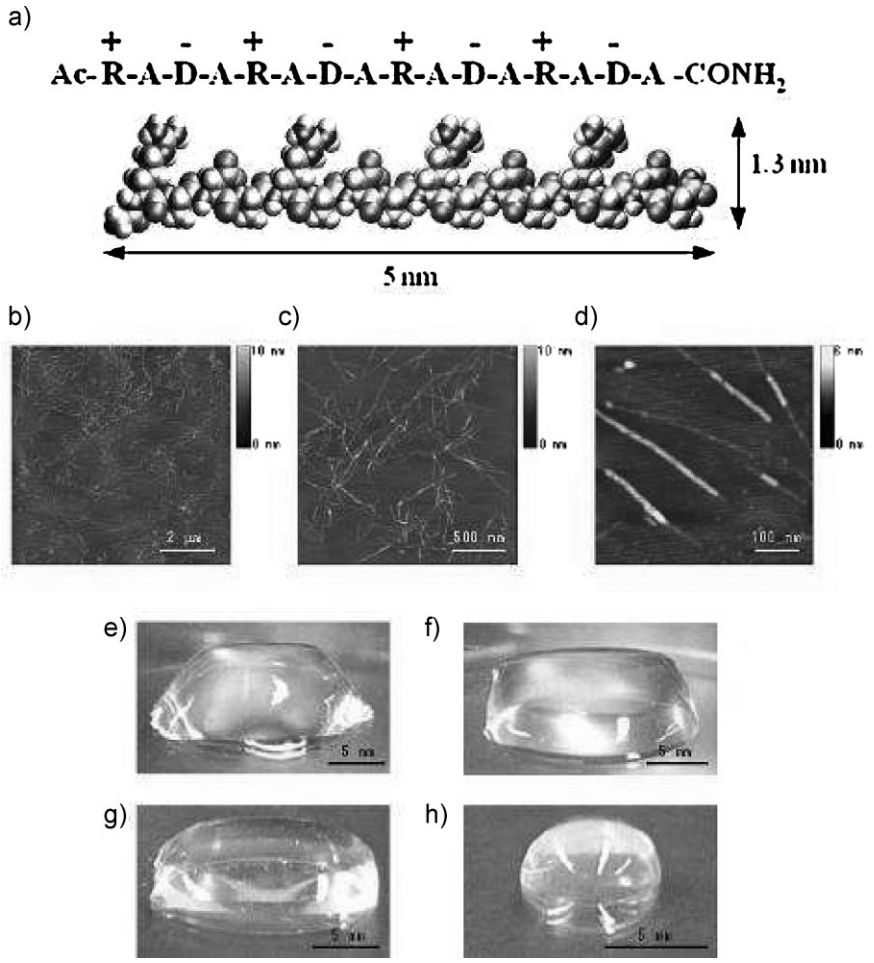

Figure 3.
Peptide RADA16-I. **a**) Amino acid sequence and molecular model of RADA16-I, the dimensions are ~5 nm long, 1.3 nm wide and 0.8 nm thick; **b**) AFM images of RADA16-I nanofiber scaffold, 8 μm × 8 μm, **c)** 2 μm × 2 μm **d)** 0.5 μm × 0.5 μm. Note the different height of the nanofiber, ~1.3 nm, in **d** suggesting a double layer structure; Photographs of RADA16-I hydrogel at various condition, **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**) re-assembled RADA16-I hydrogel after 4 times of sonication, respectively (images courtesy of Hidenori Yokoi).

through the weak interactions including hydrogen bonds, ionic bonds, hydrophobic and van der Waals interactions as well as water-mediated hydrogen bond formations. These nanofibers can be broken mechanically with sonication.[8] However, they can undergo dynamic re-assembly repeatedly (Figure 4), similar as material self-healing process. Since the driving energy of the assembly in water is not only through hydrophobic van der Waals interactions, but also the arrays of ionic interactions as well as the peptide backbone hydrogen bonds, this 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 re-assembly without addition of catalysts or through material processing, the supramolecular self-assembly and re-assembly event is likely to be wide spread in many unrelated fibrous biological materials where numerous weak interactions are involved. Self-assembly and reassembly are a very important property for fabricating novel materials, and it is necessary to fully understand its detailed process in order to design better biological materials.

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 nanometers. The kinetics of the nanofiber reassembly is followed closely at 1, 2, 4, 8, 16, 32 and 64 minutes as well as 2, 4, and 24 hours (Figure 4). The nanofiber length reassembly is as a function of time: by 2 hours, the peptide nanofibers have essentially reassembled to their original length. This

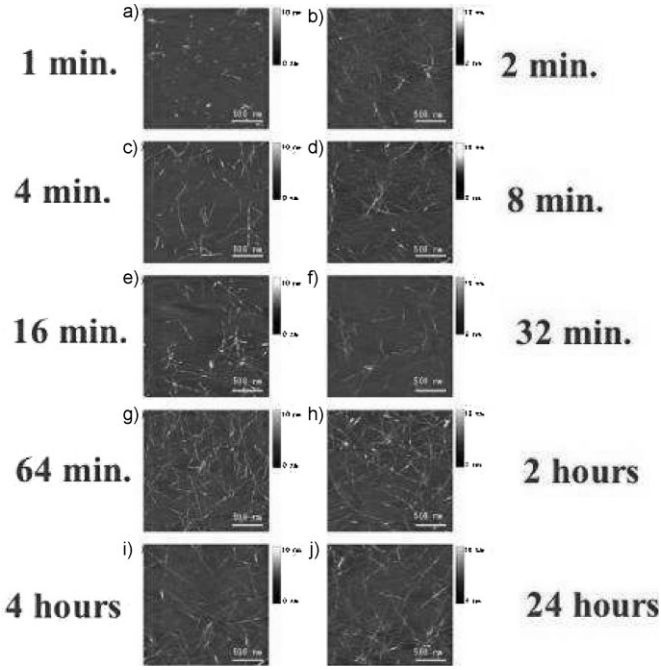

Figure 4.
AFM images of RADA16-I nanofiber at various time points after sonication. The observations were made using AFM immediately after sample preparation. **a)** 1 min after sonication; **b**) 2 min; **c**) 4 min; **d**) 8 min; **e**) 16 min; **f**) 32 min; **g**) 64 min; **h**) 2 hours; **i**) 4 hr; **j**) 24 hours. Note the elongation and reassembly of the peptide nanofibers over time. By ~1–2 hours, these self-assembling peptide nanofibers have nearly fully re-assembled (images courtesy of Hidenori Yokoi).

remarkable and rapid reassembly is interesting because there may be a little nucleation for re-growth 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 undisrupted alanine hydrophobic side that may quickly find each other (Figure 5). The situation is analogous and commonly found in sonicated and enzymatic digested DNA fragments.

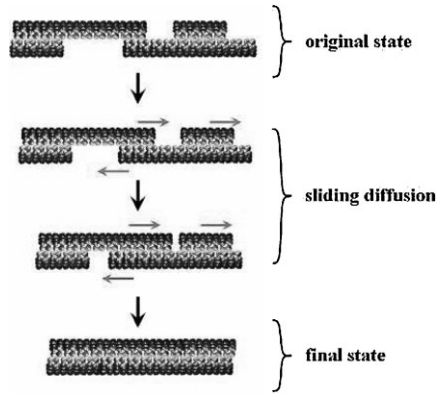

Figure 5.
A proposed molecular sliding diffusion model for dynamic reassembly of self-assembling RADA16-I peptides. When the peptides form stable β-sheets in water, they form intermolecular hydrogen bonds along the peptide backbones. The β-sheets have two distinctive sides, one hydrophobic with an array of alanines and the other with negatively charged aspartic acids and positively charged arginines. These peptides form anti-parallel 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. A proposed molecular sliding diffusion model for dynamic reassembly of self-assembling a single peptide nanofiber consisting 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 anti-parallel 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. When the fragments of nanofiber first meet, the hydrophobic sides may not fit perfectly but with gaps. However, the non-specific hydrophobic interactions permit the nanofiber to slide diffusion along the fiber in either direction that minimizes the exposure of hydrophobic alanines and eventually fill the gaps. The sliding diffusion phenomenon was also proposed for nucleic acids of polyA and polyU in 1956. For clarity, these β-sheets are not presented as twisted strands. Color code: light grey, alanines; grey, negatively charged aspartic acids; black, positively charged arginines. (Images courtesy of Hidenori Yokoi).

Kinetics of Nanofiber Reassembly and a Plausible Reassembly Process

The re-assembly kinetics is as a function of time. Perhaps, similar to DNA re-assembly, the re-assembly largely depends on the concentrations of the short complementary fragments. In this case, the fragments are the sonicated peptide nanofibers with possible presence of 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 (Figure 5). Unlike the left-handed superhelical structures observed in KFE8,[24] a different self-assembling peptide, no superhelical structures were observed for RADA16-I using AFM[8] and TEM.[5,21]

Molecular Modeling of the Self-Assembly Process

For molecular modeling clarity these RADA16-I β-sheets are presented as non-twisted 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 they also have two distinctive sides, one hydrophobic with array of overlapping alanines (Figure 5, green color sandwiched inside), similar as found in silk fibroin or spider silk assemblies.[25] The other side of the backbones have 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 non-specific, they can slide diffuse along the nanofiber, similar as trains on the train tracks. The same sliding diffusion phenomenon was also observed in nucleic acids where polyA and polyU form complementary base pairings that can slide diffuse along the chains.[26,27] If however, the bases are heterogonous, containing G, A, T, 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 due to 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, the collectively complementary + and − ionic interactions may also facilitate the reassembly. Similar to restriction-digested DNA fragments, these nanofiber fragments could form various assemblies: blunt, semi-protruding, and protruding ends. The fragments with semi-protruding and various protruding ends as well as blunt ends can reassemble readily through hydrophobic and ionic interactions (Figure 5).

Self-Assembling Peptides Nanofiber Scaffolds

The importance of nanoscale becomes obvious in 3-D cell culture. It is clearly visible in the SEM images that the cells embedded in the self-assembling peptide nanofiber biological scaffolds in the truly 3-D culture (Figure 6).[5] Here, the cells and cell clusters are intimately interact with the extracellular matrix where cells make on their own over time during cell growth and differentiation. Since the scaffolds are made mostly water, ~99% water with 1% peptide, cells can migrate freely without hindrance, similar as fish swim freely in seaweed forest.

Likewise, another self-assembling peptide KLD12 (Ac-KLDLKLDLKLDL-NH_2) was used to culture primary bovine chondrocytes (Figure 5).[22] The chondrocytes not only maintained their phenotype but also produce abundant type II collagen and glycosaminoglycans. Previously, it was known that chondrocytes dedifferentiate into fibroblast cell types and no longer produce type II collagen and glycosaminoglycans in coated 2D cell cultures. This showed the critical importance of 3D culture using the simple self-assembling peptide nanofiber scaffold.

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 (Figure 6). One example is the use of the peptide scaffolds to support neurite growth and differentiation, neural stem cell differentiation, cardiac myocytes, 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.[21] This observation stimulated and inspired further experiments to directly repair the brain of animals.

The peptide nanofiber scaffold has been used to inject into hamster's brain to reconnect the cut optical track to restore animal vision[28] because the peptide nanofiber scaffold encouraged abundant neural migration and high density synapses. In addition the same peptide nanofiber scaffold also instantly stops bleeding, ~10 seconds, in several tissues.[29] These examples are direct applications of self-assembling peptide materials for medical and clinical applications.

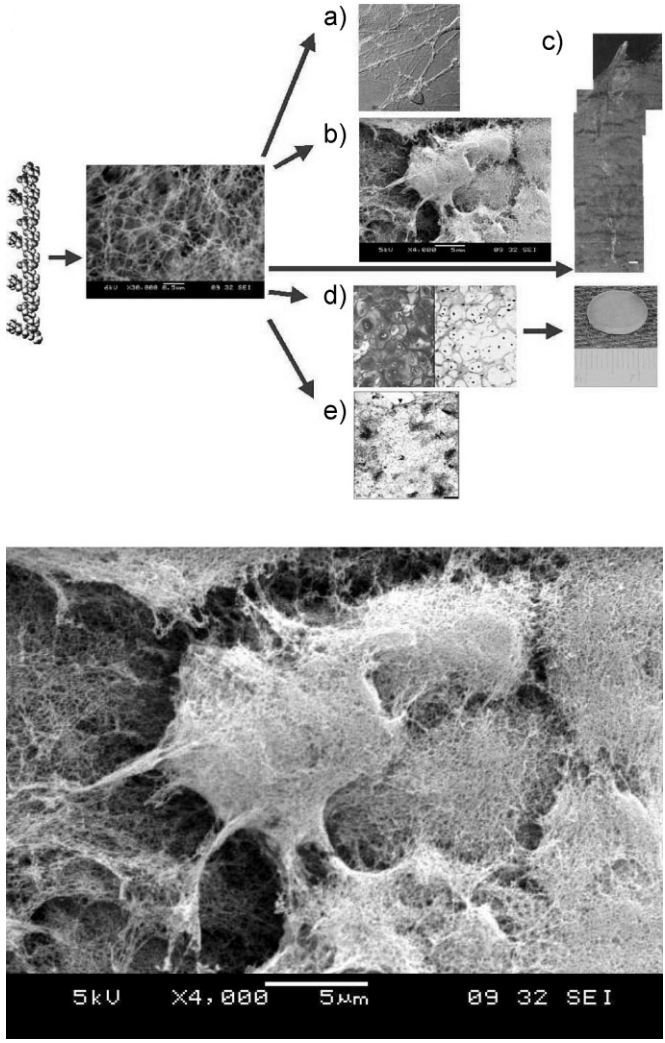

Figure 6.
From designer peptide to scaffold to tissues. **a)** Active synapses on the peptide surface. Primary rat hippocampal neurons form active synapses on peptide scaffolds. The confocal images shown bright discrete green dot labeling indicative of synaptically active membranes after incubation of 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 (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 pre-molded 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). The lower image is the cells in the 3D peptide nanofiber scaffold.

Designer Peptides Scaffold 3-D Cell Cultures

Although self-assembling peptides are promising scaffolds, they show no specific cell interaction because their sequences are not naturally found in living systems. The next logical step is to directly couple biologically active and functional peptide motifs founded in literature, accordingly the 2nd 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 it by extending the motifs on to the self-assembling peptides themselves (Figure 7). The functional motifs are coupled on the C-termini since solid phase peptide synthesis starts from C-termini to avoid deletion during elongation synthesis (the longer the peptide extended from the C-termini, the more likely there will be deletion errors). Usually a spacer comprising 2 glycines residues is added to guarantee a flexible and correct exposure of the motifs to cell surface receptors. If one combines a few designer peptides with different active motifs, these different functional motifs in various ratios can be incorporated in the same scaffold (Figure 7). Upon exposure to solution at neutral pH, the functionalized sequences self-assemble, leaving the added motifs on both sides of each nanofiber (Figure 7). Nanofibers take part to the overall scaffold thus giving microenvironments functionalized with specific biological stimuli (Figure 7).

The self-assembling peptide scaffolds with functional motifs can be commercially produced at a reasonable cost. Thus, this method can be readily adopted for wide spread uses including study how cell interact with their local- and micro-environments, cell migrations in 3-D, tumor and cancer cells interactions with the 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.[5–7] We showed that the addition of motifs in some cases to the self-assembling peptide RADA16-I did not significantly inhibit self-assembling properties, but in some case, it hindered the self-assembling process. In the latter case, the

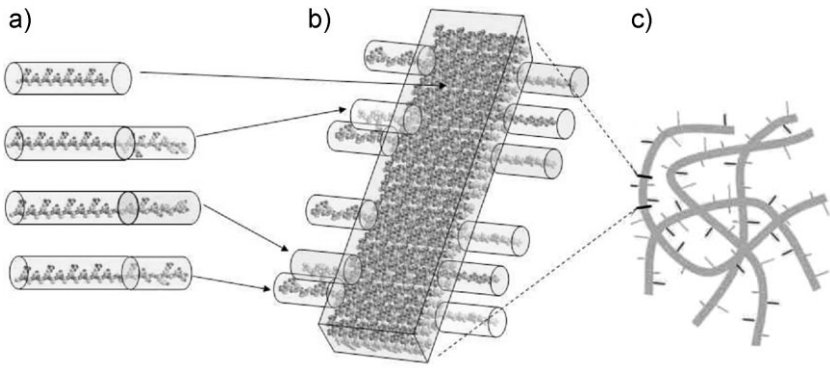

Figure 7.
Molecular and schematic models of the designer peptides and of the scaffolds. RADA16 is an alternating16-residue peptide with basic arginine, hydrophobic alanine and aspartic acid. a) Direct extension of the self-assembling peptide sequence by adding different functional motifs. Light turquoise cylinders represent the self-assembling backbone and the yellow, pink, and tan lines represent various functional peptide motifs. b) Molecular model of a self-assembling peptide nanofiber with functional motifs flagging from both sides of the double ß-sheet nanofibers. Either mono or multiple functional (or labeled) peptide motifs can be mixed at the same time. The density of these motifs can be easily adjusted by simply mixing them in various ratios, 1:1-1,000 or more before the assembling step. c) They then will be part of the self-assembled scaffold (illustration courtesy of Akihiro Horii).

peptide with active and functional motifs could be mixed in various ratios, 1:1-100 with the 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. Furthermore, it can be combined with multiple functionalities including the soluble factors [[30], also Gelain et al, submitted 2009]. 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.

In our search for additional functional motifs, we found that a class of bone marrow homing peptides BMHP[5] is one of the most promising active motifs for stimulating adult mouse neural stem cells (NSC) adhesion and differentiation.[6] This observation suggests a new class of designer self-assembling peptides for 3-D stem cell biology studies.

Designer Peptide Scaffolds for Cell 3-D Migration

The designer self-assembling peptide nanofiber scaffolds have been shown not only to be an excellent biological material for 3-D cell cultures and are capable to stimulate cell migration into the scaffold.[6,7] 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). The new peptide scaffolds are 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 pre-osteoblast 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 3D scaffold (Figure 8).[6,7] Without the modified active motif, cells stayed in the same scaffold. These observations will likely stimulate further research to study cell migration in 3D under well-defined conditions since the designer scaffolds can be fine-tuned and well controlled. This is in sharp contrast with the current cell culture conditions using collagens and Matrigel that contain unknown ingredients thus difficult to reproduce the experimental results.

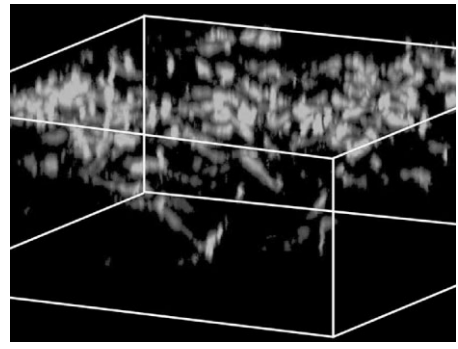

Figure 8.
Laser confocal scanning microscopy images of bone cells show the cell migrated several hundred microns into the peptide scaffold. The 3D culture system is more realistic for real body 3D environment, in sharp contrast to the conventional 2D artificial culture system (image courtesy of Akihiro Horii).

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

Designer self-assembling peptide nanofiber scaffolds have a wide spectrum of uses in additional to 3D cell culture.[32,33] Bokhari et al in UK produced a peptide hydrogel–polyHIPE polymer hybrid material to enhance osteoblast growth and differentiation.[34] Richard Lee and his colleagues at Harvard Medical School used the scaffolds to promote angiogenesis[35] and when inject into mice heart muscle, self-assembling peptide nanofibers create intramyocardial microenvironments for endothelial cells.[36] Moreover, local myocardial IGF-1 delivery with biotinylated peptide nanofibers improves cell therapy for myocardial infarction suggesting the peptide scaffolds can be used for healthcare technology.[37]

Designer Self-Assembling Peptide Nanofiber Scaffolds for Controlled and Sustained Molecular Releases

When one examines the scaffold under SEM and AFM, it is apparent that the self-assembling peptide form ordered 3D scaffold with pores range from 5-200 nanometers,[2,5] similar to size as most small molecular drugs and therapeutic proteins. This simple observation suggests that scaffolds themselves may be useful for sustained molecular release. When some small molecules are encapsulated in the peptide nanofiber scaffolds, these small molecules released slowly depending on their charges and other characteristics.[38] These observations inspired us to directly encapsulate protein molecules since proteins become increasingly effective therapeutics including insulin, and monoclonal antibodies (Figure 9).[39]

The proteins showed a sustained release mostly depend on the protein molecular size and shape. The smaller proteins, such as lysozyme with a molecular weight ~14 KD, it releases faster than antibodies which are ~10 time larger, with a molecular weight ~150 KD.[39] Furthermore, the release profiles can be fine-tuned since the lower concentrations of peptides form lower density of scaffolds, thus large pores. On the other hand, higher concentrations of peptide form higher density nanofiber scaffolds, thus having smaller pores. If amplifying 10 billion times, this situation

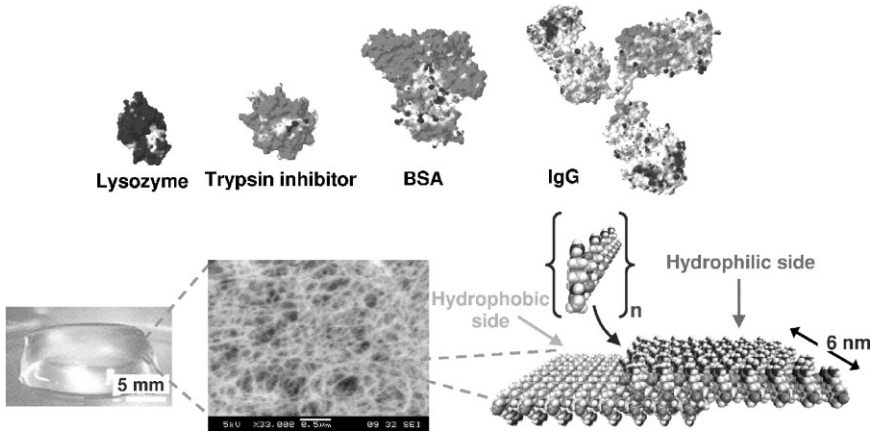

Figure 9.
Molecular representation of lysozyme, trypsin inhibitor, BSA, and IgG as well as of the ac-$(RADA)_4$-$CONH_2$ peptide monomer and of the peptide nanofiber. Color scheme for proteins and peptides: positively charged (dark grey), negatively charged (grey), hydrophobic (light dark grey). Protein models were based on known crystal structures (image courtesy of Sotirios Koutspolous).

is like a forest or grassland, the sparser the trees, shrub and grass, the faster one moves, conversely, the dense the trees, shrub and grass, the slower one moves.

Chen Pu and his colleagues also used peptide scaffold to encapsulate hydrophobic anti-cancer drug ellipticine.[40] They showed that the peptides stabilized the drug and increased its efficacy since the non-stabilized ellipticine degraded quickly thus lose its potency. One of the peptide EFK16-II stabilized neutral ellipticine molecules and ellipticine microcrystals.[40] Since many anti-cancer drugs are either not water-soluble or unstable in water, their findings could be further developed for effective anti-cancer drug delivery.

Designer Lipid-Like Peptide Surfactants

After design many ionic self-complementary peptides that form nanofibers, we ventured to design other types of self-assembling peptides. Interestingly, the first question we asked was not how to design lipid-like peptides for various applications; rather, we asked questions concerning the origin of life. We wondered what are the simplest molecules that cannot only form enclosures but also allow molecules in and out as well as increase local concentration for the primitive metabolism to take place.

We thus expanded our designs to lipid-like peptides also called surfactant peptides or peptide surfactants with similar structure and chemical properties. The key characteristics of these peptides are that they must have hydrophobic tail and hydrophilic head (Figure 10). They also have defined critical aggregation concentrations (CAC or also referred as critical micelle concentrations (CMC).[12–18] These CAC can be fine-tuned. They also undergo self-assembly to form well-ordered nanostructures including nanotubes, nanovesicles and micelles (Figure 11).[12–16]

Not only do their shape and physical structure of these lipid-like peptides resemble lipids and other organic surfactants, but their chemical properties do as well. For

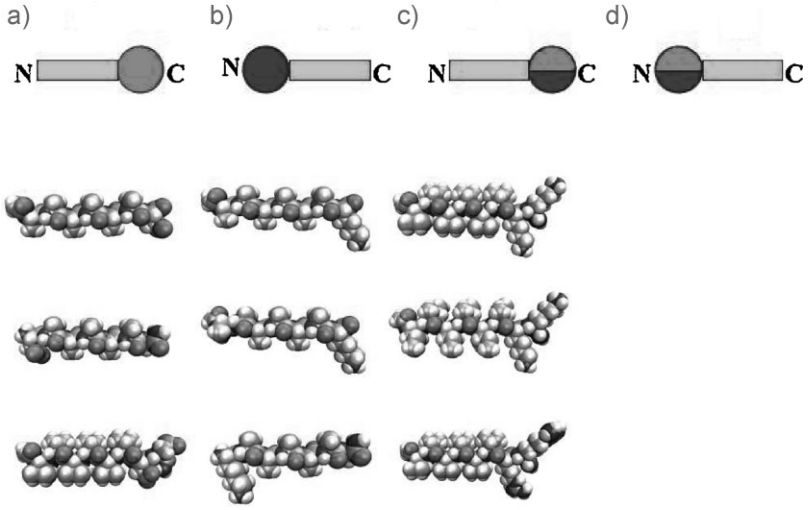

Figure 10.

The designer lipid-like peptide surfactants. A) These peptides have a hydrophilic head and a hydrophobic tail, much like lipids or detergents. They sequester their hydrophobic tail inside of micelle, vesicles or nanotube structures and their hydrophilic heads expose to water. At least three kinds molecules can be made, with $-$, $+$, $-/+$ heads. B) Six examples of designer lipid-like peptide surfactants. There are (from left to right and top down): Ac-A_6D-OH, Ac-A_6K-NH_2, Ac-V_6KK-NH_2, DA_6-NH_2, Ac-A_6K-OH, Ac-I_6KK-NH_2, Ac-V_6DD-OH, KA_6-NH_2, Ac-V_6RR-NH_2. Each one has different CAC and behaves differently in water and in PBS. They have been used to stabilize membrane protein and membrane protein complexes (image courtesy of Sotirios Koutspolous).

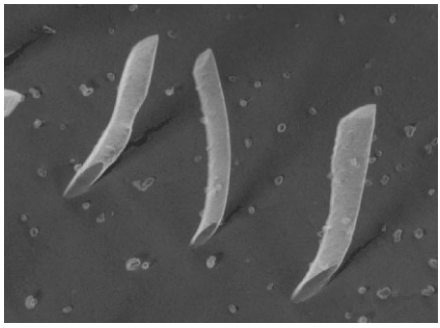

Figure 11.
TEM and AFM image of designer lipid-like peptide surfactant. These peptides undergo self-assembly to form nanotube structure. Quick-freeze/deep-etch TEM image of Ac-V_6D-OH dissolved in water (4.3 mM at pH7). The TEM images show the dimensions, ~30–50 nm in diameters with openings of nanotube ends. Note opening ends of the peptide nanotube may be cut vertically. The strong contrast shadow of the platinum coat also suggests the hollow tubular structure. The smaller spheres are likely to be the peptide micelles and vesicles (image courtesy of Steve Yang).

example, peptides have either 6 hydrophobic alanine or valine residues from the N-terminus, followed by a negatively charged aspartic acid residue (A6D = Ac-AAAAAAD-OH; V6D Ac-VVVVVVD-OH); thus, they possess two negative charges, one from the charged terminal side chain and the other from the C terminus.[12] In contrast, several simple peptides, G4DD (Ac-GGGGDD-OH), G6DD (Ac-GGGGGGDD-OH), G8DD (Ac-GGGGGGGGDD-OH), have 4, 6, and 8 glycines, followed by two aspartic acids with three negative charges.[13] Similarly, A6K (Ac-AAAAAAK-NH_2) or KA6 (KAAAAAA-NH_2) has 6 alanines as the hydrophobic tail and a positively charged lysine as the hydrophilic head.[14] These lipid-like peptides can self-organize to form well-ordered nanostructures, including micelles, nanotubes, and nanovesicles in water (Figure 11). Furthermore, the structure formation is concentration-dependent; namely, at low concentration, there are no defined structures; these structures spontaneously form at a critical aggregation concentration (CAC),[14–16] similar to lipids and other surfactants.

Six amino acids of varying hydrophobicity (Gly, Ala, Val, Ile, Leu, and Phe) can be used to generate the nonpolar tails. Such hydrophobic tails never exceed 6 residues, so that the total length of the peptide detergents will be seven, about 2.4 nm in length; interestingly, this is a similar size to that of the phospholipids abundant in membranes. The first lipid-like peptide surfactant was designed by modeling a peptide using the phosphatidylcholine as a molecular size guide.

However, when more than 6 hydrophobic residues (except glycine) are used, the peptide surfactants become less soluble in water. Although we only focused to study Asp ($^-$) and Lys (+) as the hydrophilic head groups, it must be emphasized that Glu ($^-$), Arg (+), and His (+) can also be used the same combinatorial ways. Therefore, they can broaden the spectra of variations and increase the possible number of peptide surfactants.

Design New Geometry of Lipid-Like Peptide

Besides the electrostatic and hydrophobic interactions, the dimensions and shapes of the supramolecular structures also depend on other factors, such as the geometry of the polar head group and the geometrical constraints of the peptide itself.[18] Thus, it is possible to fine-tune the supramolecular structures with expanded functionalities of peptide surfactants by introducing different shapes and structures. The donut-shaped nanostructure formation was observed from self-assembly of a cone-shaped designer lipid-like peptide.

The cone-shaped amphiphilic peptide, Ac-GAVILRR-NH_2, has a hydrophilic head with two positive charges and with a relatively large size, and has a hydrophobic tail with decreasing hydrophobicity and side-chain size (Figure 12). The CAC values were measured using dynamic light

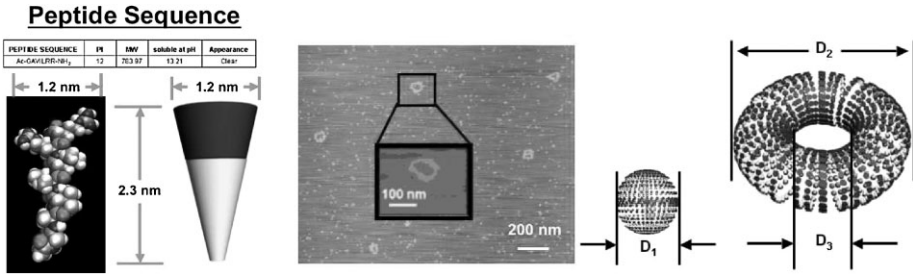

Figure 12.
Molecular models and self-assembled donut-shaped structure of Ac-GAVILRR-NH_2. The peptide length is approximately 2.3 nm and the width is 1.2 nm. Color code: hydrogen = white, carbon = cyan, oxygen = red and nitrogen = blue. The cone-shaped model is simplified for the shape of Ac-GAVILRR-NH_2. The blue part indicates the positively charged hydrophilic region and the yellow part indicates the hydrophobic region. AFM image of the donut structure of the self-assembled Ac-GAVILRR-NH_2 is presented (image courtesy of Ulung Khoe).

scattering in water (0.82 mM) and in phosphate buffered saline (0.45 mM) as well. The AFM image in Figure 8b shows the typical donut-shaped nanostructures of the cone-shaped peptide assembly at a concentration of 1 μM. We have studied numerous peptides over 17 years[3] and this is the first time ever we observed the nanodonut structure from the cone-shaped peptides. The dimensions of the donut-shaped assemblies were measured using the section analysis. The measured outer diameter $D_2 = \sim 105$ nm, and the inner diameter $D_3 = \sim 22$ nm. The average thickness of the donut-shaped structures is around ~41.5 nm, which is similar to the diameter of the spherical nanoparticles. The schematic illustration of the nanodonut structure is presumed to be the elongated micelle and curved to be a closed ring.

Interestingly, AFM observations of some short nanopipe structures with different bending angles are captured in a single image.[18] Based on this observation, we propose a plausible self-assembling pathway of the nanodonut structure that was self-assembled through fusion or elongation of spherical micelles. Furthermore the bending of the nanostructure gives rise to the nanodonut structures due to the tension originated from the interaction of the cone-shaped peptide side-chains. Our observations may be useful for further fine-tuning of the geometry and shape of a new class of designer peptides and their self-assembled supramolecular materials for diverse uses. This unique cone-shaped geometry would not only add repertoire of the self-assembling nanostructures but also deepen the understanding of the mechanisms of the peptide self-assembling.

One of the questions that were frequently asked is if the lipid-like peptide can indeed mix well with real lipid molecules. Experiments using monoolein bilayers to incorporate the designer lipid-like peptides showed that these peptides could indeed interact with the monoolein very well.[44] The impact of these lipid-like peptide surfactants and the ternary MO/peptide/water system has been studied using small-angle X-ray scattering (SAXS). At higher peptide concentration (R = 0.10), the lipid bilayers are destabilized and the structural transition from the Pn3m to the inverted hexagonal phase (H(2)) is induced. For the cationic peptides, our study illustrates how even minor modifications, such as changing the location of the head group Ac-A_6K-NH_2 vs. KA_6NH_2 affects significantly the peptide's effectiveness. Only KA_6NH_2 displays a propensity to promote the formation of H(2), which suggests that KA_6NH_2 molecules have a higher degree of incorporation in the interface than those of Ac-A_6K-NH_2.[44]

Designer Lipid-Like Peptide Stabilize Membrane Proteins

Membrane proteins play vital roles in all living systems. They are crucial for biological energy conversions, cell-cell communications, specific ion channels and pumps involving our senses: sight, hearing, smell, taste, touch and temperature sensing. Approximately ~30% of all genes in almost all sequenced genomes code for membrane protein.[45–47] However, our understanding of their structures and function falls far behind that of soluble proteins. As of November 2009, there are only 211 unique membrane protein structures of total 566 variations known (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html) among over 61,000 structures in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do), The reason is that there are several notoriously difficult steps to obtain membrane proteins.

Membrane proteins are exquisitely fine nature-made molecular devices that will be very useful for a wide range of applications including solar energy harvesting and ultrasensitive sensing. In order to accelerate membrane protein structural studies and use them for design and fabrication of nanobiodevices, new and simple methods are crucial. We found that simple lipid-like peptides are excellent materials to solubilize and stabilize these proteins.

Detailed structural analyses of membrane proteins as well as their uses in advanced nanobiotechnology applications require extended stabilization of the functional protein conformation. We have shown that the designer surfactant-like peptides can significantly increase the activity and stabilize diverse membrane

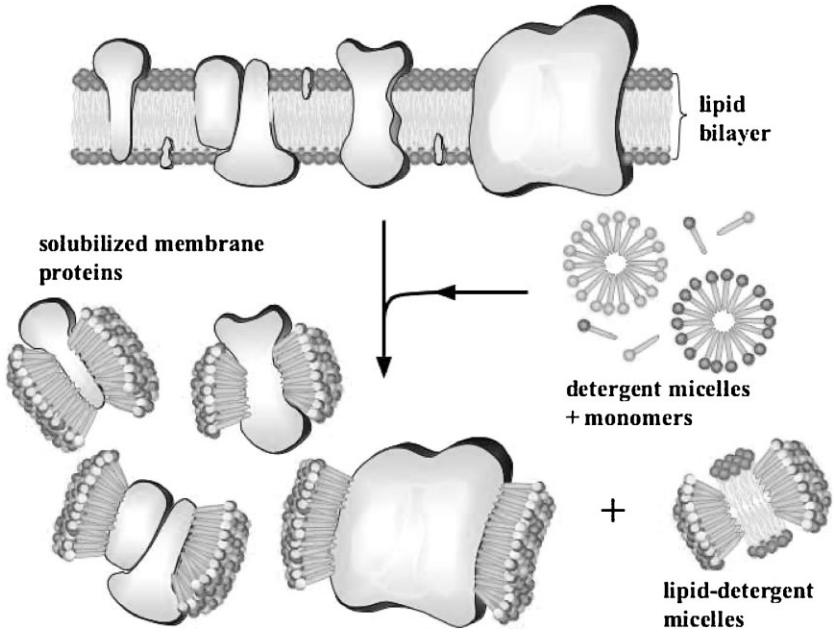

Figure 13.
A proposed scheme for how the designer lipid-like peptides stabilize membrane proteins. These simple designer self-assembling lipid-like peptides have been used to solubilize, stabilize and crystallize membrane proteins. These peptides have a hydrophilic head and a hydrophobic tail, much like other biological lipids. They use their tail to sequester the hydrophobic part of membrane proteins, and the hydrophilic heads exposed to water. Thus, they make membrane proteins soluble and stable outside of their native cellular lipid milieu. These lipid-like peptides are very important for overcoming the barrier of high resolutions of molecular structure for challenging membrane proteins.

proteins including *E.coli* Glycerol-3-phosphate Dehydrogenase,[48] G-protein coupled receptor, for example the bovine rhodopsin,[49] the functional form of the multi-domain protein complex Photosystem-I (PS-I) on surface in dry form[50] and in aqueous solution.[51] We carried out a systematic analysis using a series of such peptides to identify the chemical and structural features that enhance the photochemical activity of PS-I. We observed that lipid-like peptide surfactant amphiphilicity is necessary, but not sufficient to stabilize PS-I in its functional form. Furthermore, we showed that not only the polarity and number of charges on the hydrophilic head are important, but also the hydrophobicity and size of the amino acid side groups in the hydrophobic tail play an important role. For bovine rhodopsin, A6D-OH is the best.[49] For Photosystem-I the best performing peptides for the stabilization of functional PS-I are in order of effectiveness: ac-I_6K_2-$CONH_2$, ac-A_6K-$CONH_2$, ac-V_6K_2-$CONH_2$, ac-V_6R_2-$CONH_2$.[51] These simple and inexpensive peptide surfactants will likely make significant contributions to stabilize the functional form of diverse and currently elusive membrane proteins and their complexes with important applications.

A Hierarchical α-Helical Self-Assembling Peptide

We not only designed numerous β-sheet forming peptides, lipid-like peptides, secondary structural transitional peptides, but we also designed a helical coiled-coil hierarchical self-assembling peptide that self-organized into fractal structure[52] that fulfills the fractal definition of self-similarity at multiple length scales. In this design, the hierarchical self-assembling peptide with a cross-linkable coiled-coil contains an internal cysteine. The fractal structure of the hierarchical assemblies can be seen using Atomic force microscopy (AFM), low-power optical microscope and unaided-eyes. Molecular simulations showed that the peptides cross-linked to form clusters of coiled-coils, which further assembled to form globules of tens of nanometers in diameter. The hierarchical organization is modulated by pH or thiol-reducing agent. Exploitation of the fractal structures through chemical methods may be valuable for the fabrication of materials spanning multiple length scales with a wide range of applications.

Conclusion

The word ''designer'' is often associated with designer watches, sunglasses, hang bags, shoes, clothes, and other luxury commercial goods. There, the designers have absolutely control for the ultimate outcome of their products, from the selection of the materials to the look and feel of the products. They are the masters of the designer's world. Now, we can do the same at the molecular scale. We can design the new materials at the single molecular level, in some cases, single atom level. The macromolecular chemistry is to design the individual parts and allow the self-assembly of numerous individual parts into a coherent whole and sometimes, with functional entity. Nature has done it for billions of years with elegance and simplicity, now it is time we follow Nature's leads.

Leonardo da Vinci stated over 550 years ago ''When nature finishes to produce its own species, man begins using natural things in harmony with this very nature to create an infinity of species''. Although we have created many molecular species using natural amino acids, there is no doubt, infinite more will come.

Susan Lindquist of MIT also said eloquently, ''About 10000 years ago, human begin domestic plants and animals. Now it's time to domestic molecules''. Indeed, we are just at the beginning to domestic and design numerous totally new molecules, new biological materials with defined and desired functionalities.

The human civilizations are usually divided according to the materials that dominate in the society: the Stone Age, the

Bronze Age, the Iron Age, the Plastic age and the Silicon age. The new designer materials age is now upon us. The designer biological materials space is widely open; the only limit is our limited imaginations.

We believe that these simple and versatile designer self-assembling peptides will provide us many opportunities to study complex and previously intractable biological phenomena. Molecular engineering through designer self-assembling peptides is an enabling technology that will play an increasingly important role in biomedical research, bioengineering, nanobiotechnology, clinical medicine and beyond.

A Few Words for Students and Young Researchers

In pursuing frontier and original research, it is not infrequent that one encounters unexpected observations and experiments that mystify the observer since no one has reported it before. If the observations and the experiments are reproducible with all necessary controls, then one must choose either ignore it, or continue to pursue what is the funded project, or to pursue the unexpected observation relentlessly to find the answers. Most people would choose the former since it is funded, and it is the goal of the research to satisfy the assigned project and the granting agencies. A few daring and risk-takers may chase the unexpected observations persistently. Perhaps most people would consider that this is chasing the rainbow. There are many difficulties, set backs, unaccepted by colleagues and peers. Manuscripts are ridiculed and rejected. The grant applications are turned down repeatedly. It takes a lot of courage to overcome peer pressure and eventually to persuade colleagues and peers that the unexpected observations are worthwhile to pursue with merit. There are many such examples in scientific discoveries including Nicolaus Copernicus' scientifically-based heliocentric cosmology that displaced the Earth from the center of the universe; Charles Darwin's theory of evolution; Alfred Wegener's plate tectonics; DNA as the genetic material, Alexander Rich's discovery of DNA-RNA and RNA-RNA perfect pairing without enzymes, Benoit Mandelbrot's discovery of the Fractals; Carleton Gajdusek's discovery and finding the solution for the seemingly incurable Kuru disease, Thomas Cech's discovery of RNA self-splicing, Victor Ambros and Gary Ruvkun's discovery of mircoRNA, and many more. The resistance from the establishment is very strong. One must trust one's own reproducible experiments and careful observations, thus one must be completely self-confident and persistent.

Since we started the serendipitous journey of working on designer self-assembling peptide systems, we have encountered many surprises, from developing a class of pure peptide nanofiber scaffolds for 3-D tissue culture and regenerative medicine, to design lipid-like peptides that solubilize, stabilize and crystallize membrane proteins, to study the model system of protein conformational diseases. As Nobel laureate D. Carleton Gajdusek best put it "*It is important to explore, to do things others ignore but that will become important in 10-20 years*".

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