Self-assembling peptide materials

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1 Introduction

1.1 Discovery of the first self-assembling peptide

While working on yeast genetics and protein chemistry and trying to understand a left-handed Z-DNA structure in the laboratory of Alexander Rich at Massachusetts Institute of Technology in 1989, I identified a protein *Zuotin* (Zuo in Chinese means left and there is a Z in it) for its ability to bind to left-handed Z-DNA in the presence of 400-fold excess of sheared salmon DNA that contains ubiquitous right-handed B-DNA and other form DNA structures, in it, there is a repeat segment with the sequence AEAEAKA-KAEAEAKAK, thus I named EAK16 for its amino acid composition and peptide length (Fig. 1). In the yeast zuotin, the sequence repeats 3 times.[1]

Initial computer modelling of this EAK16 sequence showed the structure to be a α-helix: its lysines and glutamic acids on the side-chains with *i, i + 3* and *i, i + 4* arrangements could form potential ionic bonds. As a curiosity, I wondered if this peptide could be synthesized and studied to satisfy my scientific curiosity. At the time, I could not at all predict that my curiosity led me into an entirely unexpected field of peptide materials, now thriving and many products and applications have been realized in diverse fields.

Alexander Rich who always has an open mind then strongly supported me to order the actual EAK16 peptide. For economic reasons, we ordered a custom synthesis through the Biopolymers Laboratory at Massachusetts Institute of Technology.

When the EAK16 peptide was first studied following the reported method using circular dichroism spectroscopy, an unexpected result occurred, instead of showing the expected computered modelled α-helix, the peptide formed an exceedingly stable β-sheet structure. Upon adding salt, a thin layer membrane-like substance and transparent materials occurred in the petri dish.[2,3]

I later met the late legendary Francis Crick and told him about the discovery. Crick suggested me to look under scanning electron microscope (SEM), as I did. It took me more than one year to understand how the seemingly soluble short peptides underwent self-assembly to form well-ordered nanofibers and scaffolds and to form naked-eye visible materials. I and my colleagues published the yeast *Zuotin* where the first self-assembling peptide that formed visible material was discovered.[1] Since then self-assembling peptide has been expanded in a number of directions in past two decades.[4–13]

1.2 Self-assembling peptide materials

Designer materials that are self-assembled molecule by molecule or atom by atom to produce novel supramolecular architectures belong to

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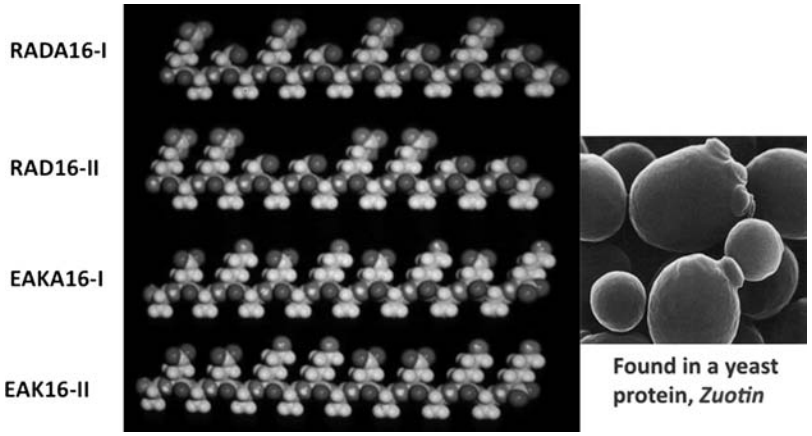

Fig. 1 The simple and molecular models of the designer amphiphilic self-assembling peptides that form well-ordered nanofibers. These peptides have two distinctive sides, one hydrophobic and the other hydrophilic. The hydrophobic side forms a double sheet inside of the fiber and the hydrophilic side forms the outside of the nanofibers that interacts with water molecules, forming an extremely high water content hydrogel that contains as high as 99.9% water. At least three types of molecules can be made, with $-$, $+$, $-/+$ on the hydrophilic side. The individual self-assembling peptide molecules are $\sim$5 nm long. The first such peptide, EAK16-II, was discovered from a yeast protein, zuotin.[1,2] This peptide inspired us to design a large class of self-assembling peptide construction motifs. When dissolved in water in the presence of salt, they spontaneously assemble into well-ordered nanofibers and then further into scaffolds.

"bottom-up" instead of "top-down" approach, and likely become an integral part of materials manufacture. This approach requires a deep understanding of individual molecular building blocks, their structures and dynamically assembly properties.[4,5]

These self-assembling peptides have alternating hydrophobic, namely, alanine, valine, leucine, isoleucine, and phenylalanine, and hydrophilic sides, which include positively charged lysine, arginine, histidine and negatively charged aspartic acids and glutamic acids.[4,5]

The complementary ionic sides have been classified into modulus I, II, III, IV and mixed moduli. This classification is based on the hydrophilic surface of the molecules that have alternating $+$ and $-$ charged amino acid residues, either alternating by 1, 2, 3, 4 and so on. For example, charge arrangements for the different moduli are as follows: modulus I, $-+-+-+-+$; modulus II, $--++\ \ --++$; modulus III, $---+++$; and modulus IV, $----++++$ (Fig. 2). The charge orientation can also be designed in reverse orientations that yield entirely different molecules with distinct molecular behaviors. These well-defined sequences allow them to undergo ordered self-assembly, resembling some situations found in well-studied polymer assemblies. This simple idea is the basis of the self-assembling peptide building blocks.

1.3 Basic chemical properties of the self-assembling peptide systems

The peptide synthesis has become more and more affordable that uses conventional mature solid phase or solution peptide synthesis chemistry. The peptide production cost directly correlates with the motifs length, purity of peptides, skill of the manufactures and the chirality of amino

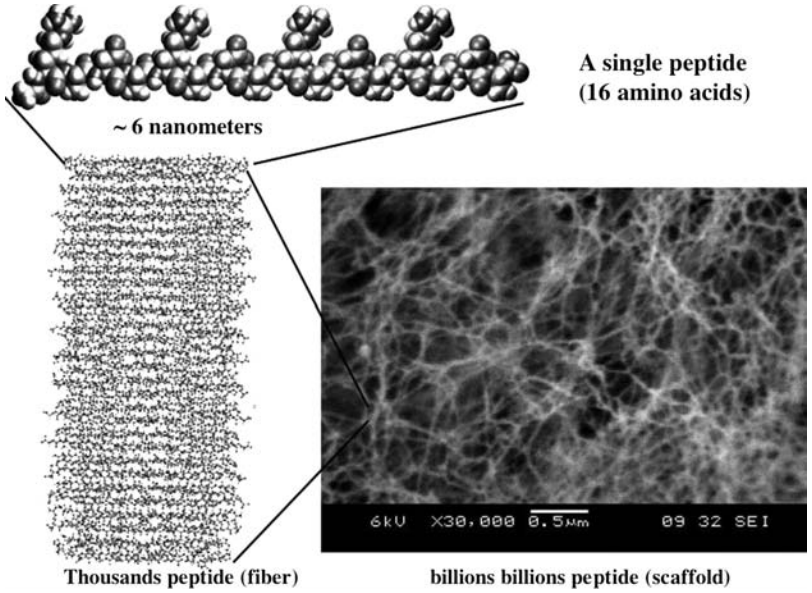

Fig. 2 Self-assembling peptide RADA16-I nanofiber scaffold hydrogel. Upper panel) Amino acid sequence of RADA16-I, molecular model of a single RADA16-I nanofiber, the calculated peptide dimensions are ~6 nm long depending on end capping, 1.3 nm wide and 0.8 nm thick; tens and hundreds of thousands of individual peptides self-assemble into a nanofiber, SEM images of RADA16-I nanofiber scaffold. Note the scale bar, 0.5 μm or 500 nm (SEM image courtesy of Fabrizio Gelain). RADA16-I Peptide form nanofibers in aqueous solution that further form hydrogel with extremely high water content (99.5–99.9% w/v water).

acids. Most of self-assembling peptides are readily soluble in water since their amino acid molecules consist of alternating hydrophilic and hydrophobic that contain 50% charged residues with distinct polar and non-polar surfaces and periodic repeats of 2–4 times. The self-assembly is accelerated by millimolar salt concentration under the physiological pH conditions to form ordered-nanostructure such as nanofiber, nanotube and nanovesicle.[2–8]

For example, RADA16-I and RADA16-II with arginine and aspartate residues replacing lysine and glutamate have been designed. The alanines form overlapping hydrophobic interactions in water, both positive Arg and negative Asp charges are packed together through intermolecular ionic interactions in a checkerboard-like manner. They self-assemble to form nanofibers ~10 nm in diameter, and theses nanofibers interwove into scaffolds that retain extremely high hydration, >99% in water (1 mg–10 mg/ml, w/v) (Fig. 3).[14]

The formation of the scaffold and its mechanical properties are influenced by several factors 1) amino acid sequence, 2) the level of hydrophobicity 3) length of the peptides, and 4) self-assembling time. For example to 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.[15]

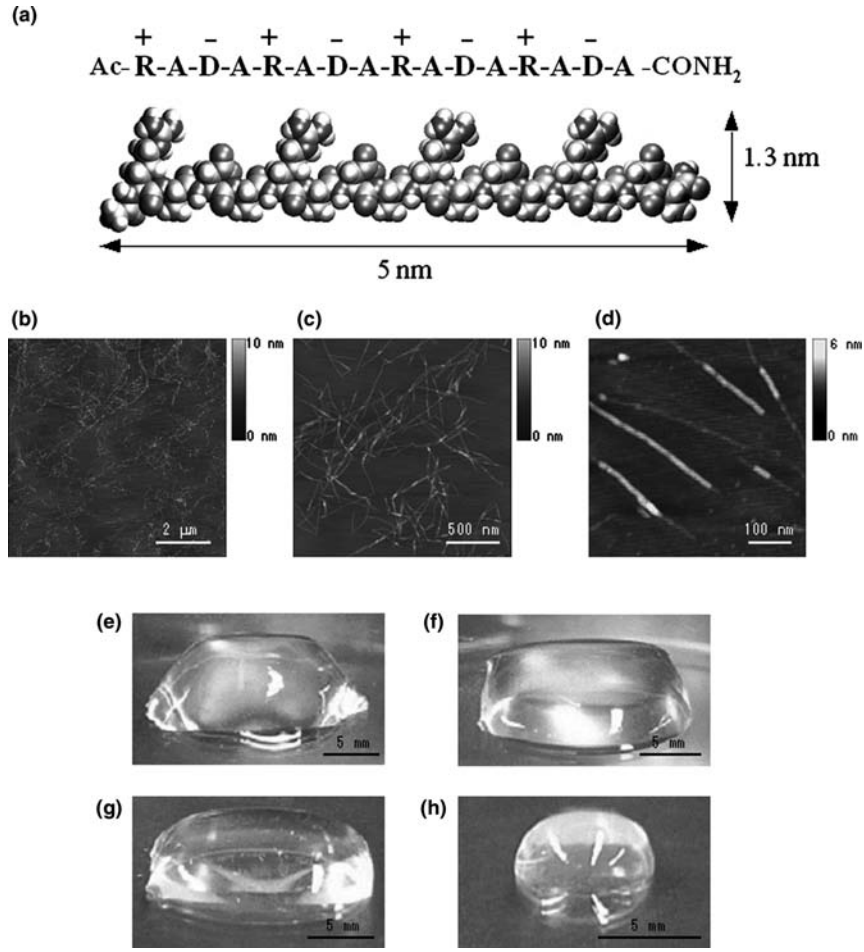

Fig. 3 Peptide RADA16-I. **a**) Amino acid sequence and molecular model of RADA16-I, the dimensions are ~5 nm long, 1.3 nm wide and 0.8 nm thick; **b**) AFM images of RADA16-I nanofiber scaffold, 8 μm^2, **c)** 2 μm^2 **d)** 0.5 μm^2. Note the different height of the nanofiber, ~1.3 nm, in **d** suggesting 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 (image courtesy of Hidenori Yokoi).[14]

1.4 The proposed general assembly of nanofiber formations

The detailed mechanism how self-assembling peptides self-organize themselves in water at such low concentration still remains inadequately understood at present, nevertheless various peptides as biological materials have found diverse applications.

Several plausible ideas for different nanostructures have been proposed. First, for nanofibers, a molecular model to interpret the formation of EAK16 and RADA16 was proposed. These two peptides are representative of a class of peptides that undergo self-assembly into ordered-nanofibers: 1) numerous intermolecular hydrogen bonds between the peptides -C=O$\cdots\cdots$H-N- in the conventional β-sheets on the peptide backbones, 2) the side chains of positively and negatively charged residues form

intermolecular ionic bonds in a checkerboard manner, 3) hydrophobic interactions between the peptides from the amino acid residues pused by water molecules, 4) alternating polar and nonpolar surface interaction. It is known that salt ions facilitate the self-assembly, however it is not yet clear where the monovalent ions may coordinate the charged residues in a higher order of geometry.[14]

1.5 Dynamic reassembly of self-assembling peptides

The self-assembling peptides form stable β-sheet structure in water. The interactions between the peptides and β-sheets are 1) non-covalent hydrogen bonds along the backbones, 2) the arrays of ionic + and - charge interactions, 3) alanine hydrophobic interactions and van der Waals interactions, and 4) water-mediated hydrogen bond formations. Thus the nanofibers can be disrupted mechanically using sonication.[14] The self-assembling process is reversible and dynamic since these peptides are short and simple. Numerous individual peptides can be readily self-organized through the weak interactions. However, they can undergo dynamic reassembly repeatedly (Fig. 4), similar to the 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.[14]

Unlike processed polymer microfibers in which the fragments of polymers cannot undergo reassembly without addition of catalysts or through material processing, the supramolecular self-assembly and reassembly 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 and to improve biological materials.

AFM images reveal 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 closely followed at 1, 2, 4, 8, 16, 32 and 64 minutes as well as 2, 4, and 24 hours (Fig. 4). The nanofiber length reassembly is as a function of time: by 2 hours, the peptide nanofibers have essentially reassembled to their original length. The β-sheet structure had little change since the β-sheets at the molecular level remain unchanged despite the nanofiber length change.[14]

1.6 Molecular modeling of the self-assembly process

We carried out molecular modeling of the self-assembly process. For molecular modeling clarity, the RADA16-I β-sheet is presented as a non-twisted strand. 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 (Fig. 5, green color sandwiched inside), similar to what is found in silk fibroin or spider silk assemblies. The other side of the backbones has negatively charged (−)

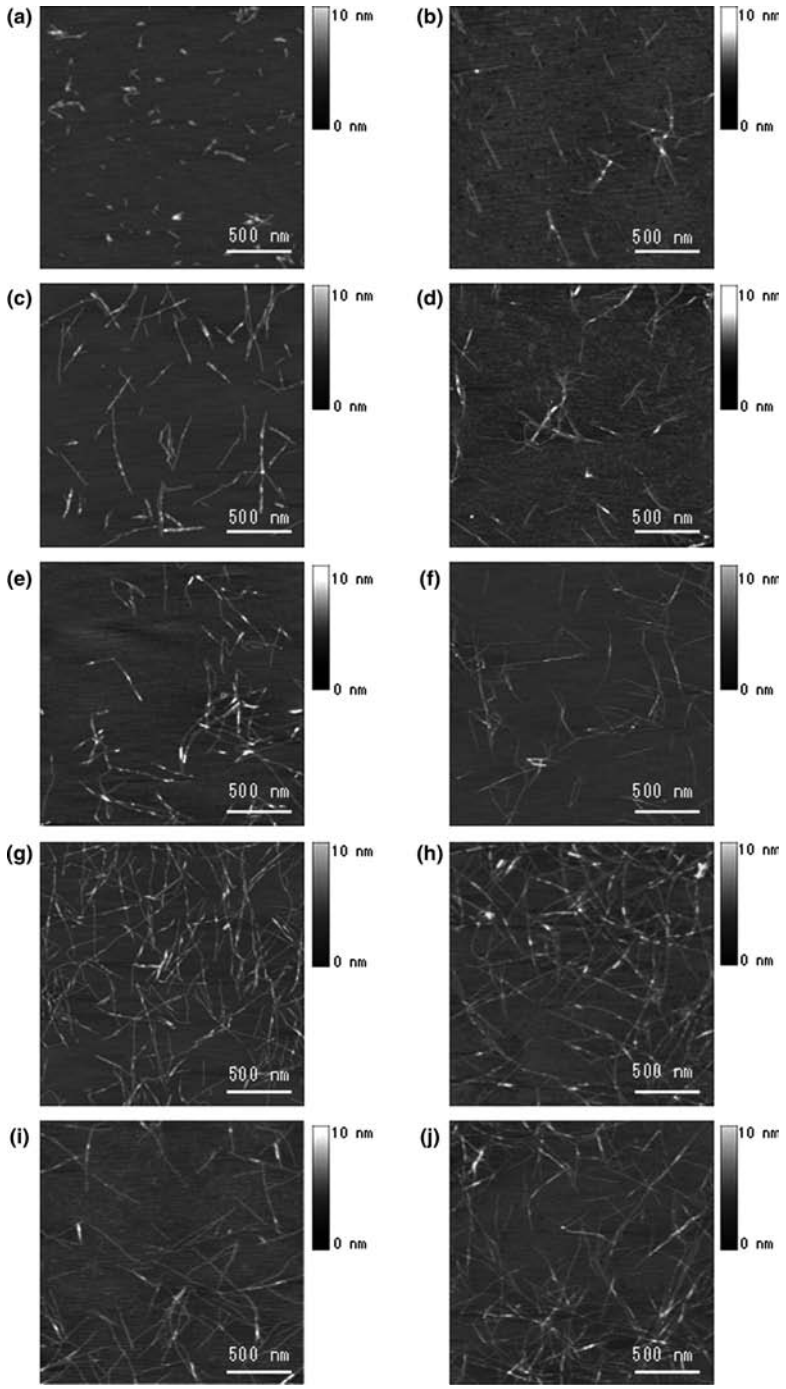

Fig. 4 AFM images of RADA16-I nanofiber at various time points after sonication. The observations were made using AFM immediately after sample preparation. **a)** 1 min after sonication; **b**) 2 min; **c**) 4 min; **d**) 8 min; **e**) 16 min; **f**) 32 min; **g**) 64 min; **h**) 2 hours; **i**) 4 hours; **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 (image courtesy of Hidenori Yokoi, PNAS).[14]

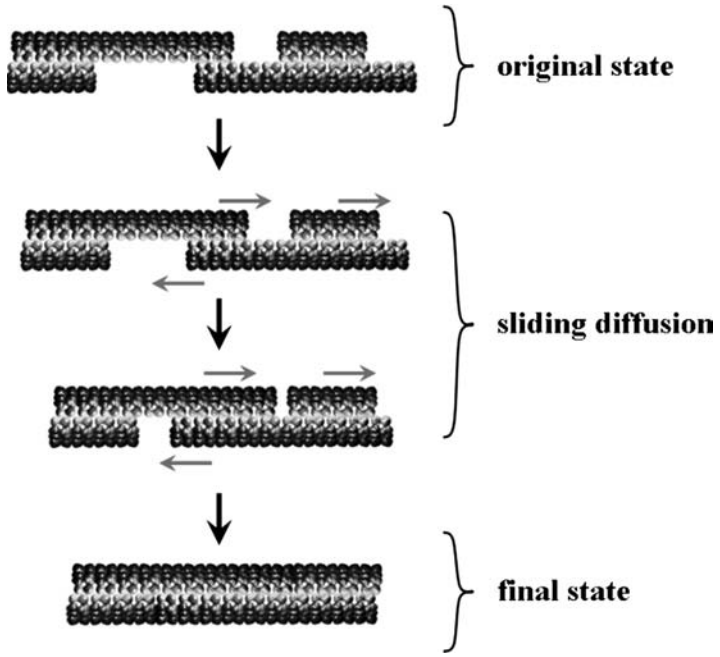

Fig. 5 A proposed molecular sliding diffusion model for dynamic reassembly of a single peptide nanofiber consisting thousands of individual peptides. When the peptides self-assemble into stable β-sheets in water, they form intermolecular hydrogen bonds along the peptide backbones. The β-sheet structure has two distinctive sides, one hydrophobic with an array of alanines and the other with negatively charged and positively charged amino acids. 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.[15–16] For clarity, these β-sheets are not presented as twisted strands. Color code: green, alanines; red, negatively charged amino acids; blue, positively charged amino acids [Colour image available on-line] (image courtesy of Hidenori Yokoi).[14]

amino acids, represented as red, and positively charged (+) amino acids, represented as blue.

The alanines form packed hydrophobic interactions in water, which can be disrupted mechanically during sonication. 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, like trains sliding along train tracks. The same sliding diffusion phenomenon was also observed in early studies of nucleic acids where polyA and polyU form complementary base pairings that can slide diffuse along the double helical chains.[15,16] If however, the bases are heterogonous, containing G, A, T, C, then the bases cannot undergo sliding diffusion. Likewise, if the hydrophobic side of the peptides does not always contain alanine, containing residues such as valine and isoleucine, it would become more difficult for sliding diffusion to occur due to their structural constraint.

On the charged side, both positive and negative charges are packed together through intermolecular ionic interactions in a checkerboard-like manner. Likewise, the collectively complementary + and − ionic interactions may also facilitate reassembly. Similar to restriction-digested DNA fragments, these nanofiber fragments could form various assemblies like blunt and protruding ends. The fragments with various protruding ends as well as blunt ends can reassemble readily through hydrophobic and ionic interactions (Fig. 5).

2 General self-assembling peptide materials

There are the general peptide materials that are generic with no specific biological active motifs, in other words, not tailor-made for specific purpose. In an analogy, the general peptide materials are like pieces of bread without butter, cream spread or jam; or a bowl of pure rice without any added flavors.

There are also several classes of peptide mateirals, 1) like Legos, they self-assemble to form nanofibers; 2) like lipids or surfactants, they self-assemble into nanotubes and nanoviescles; 3) like biological paint, they self-assemble on surfaces to modify the surfaces.

2.1 Self-assembling peptide nanofibers

The first self-assembling peptide EAK16 and RADA16 are general peptides without active motifs. Here, the RADA16 made first milestone for new economic development. RADA16 has become commercial products for various 3D tissue cell cutlures (BD Biosciences, http://www.bdbiosciences.com/). It has also been successfully developed for various applications as medical devices.

The peptides form the stable second structures including α-helix and β-sheet. But some peptides secondary structures are more dynamic under various environmental factors: i) the amino acid sequence arrangements (even with the identical composition), ii) the molecular size of the peptide, iii) peptide concentration, iv) pH of the solution, v) temperature, vi) the medium composition, such as solvent or substrate, vii) ionic strength, and viii) the presence of denaturation agents, such as sodium dodecyl sulfate (SDS), urea and guanidium hydrochloride. These factors can significantly influence the dynamic behaviours of peptide secondary structures and also affect the process of self-assembly.[2–4]

2.2 Specific self-assembling peptide materials

Although self-assembling peptides are promising materials, they show no specific cell interaction because their sequences are not naturally found in living systems. In order to introduce the specific needs for individuals, the next logical step is to tailor-make the materials by directly coupling biologically active and functional peptide motifs onto the generic peptide (Fig. 6). Accordingly, the second generation of designer scaffolds will have significantly enhanced interactions with cells and tissues as well as other molecules.

We have made a wide range of designer self-assembling peptide nanofiber scaffolds for both 3D tissue cell cultures and sustained molecular releases.[16–23]

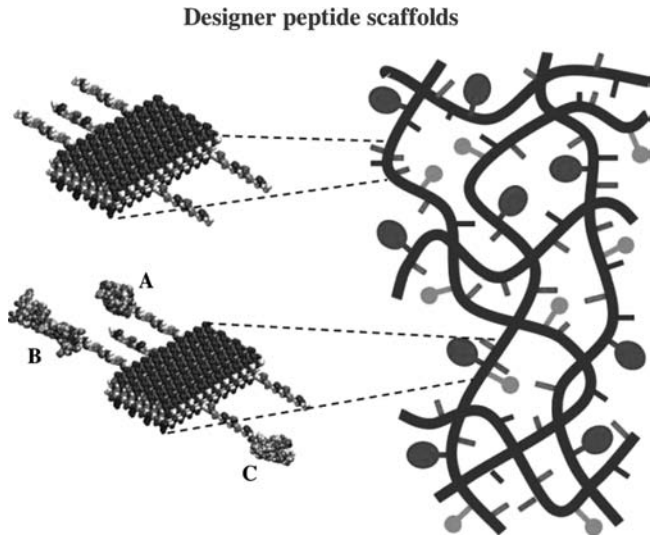

Fig. 6 Molecular and schematic models of the designer peptides and of the scaffolds. Direct extension of the self-assembling peptide sequence by adding different functional motifs. Light turquoise cylinders represent the self-assembling backbone and the yellow, pink, and tan lines represent various functional peptide motifs. Molecular model of a self-assembling peptide nanofiber with functional motifs flagging from both sides of the double b-sheet nanofibers. Either few or more functionalized and active peptide can be mixed at the same time. The density of these functionalized peptides can be easily adjusted by simply mixing them in various ratios, 1:1-1,000,000 or more before the assembling step. They then will be part of the self-assembled scaffold.[17] [Colour image available on-line]

The designer self-assembling peptides with additional active motifs play much more important and biological role and attractive biological materials with added functionalities and added values. Importantly, although additional active motifs were appended to the generic peptide backbone, the nanofiber structures remain quite similar as shown by SEM (Fig. 7).[17] Interestingly, the nanofiber structure is similar to the widely used Matrigel except the Matrigel is not pure with many other substances and growth factors in it. In some cases, when the active sequences become longer, the self-assembling speed and mechanical property are affected.[18–19,21–22] The 3D nanofiber materials are superb scaffolds for diverse tissue cells, not only for differentiation, proliferation, migration, but also for long-term maintenance.[17–19,21–22]

In order to fully understand how cells behave such as the 3-dimensional (3-D) microenvironment, 3-D gradient diffusion, 3-D cell migration and 3-D cell-cell contact interactions and in tissue engineering and regenerative medicine, it is important to develop a well-controlled 3-D tissue culture system where every single ingredient is known.

For over 100 years since the Petri dish was invented and used for tissue culture studies, almost all tissue cells have been studied on the 2D Petri dish and various formats of coated 2D surfaces. However, this 2D surface is rather unlike 3D tissue and the body's microenvironment. Thus it is important to develop a true 3D microenvironment to mimic the real tissue and body situation. The commonly used biomaterials are inadequate due to

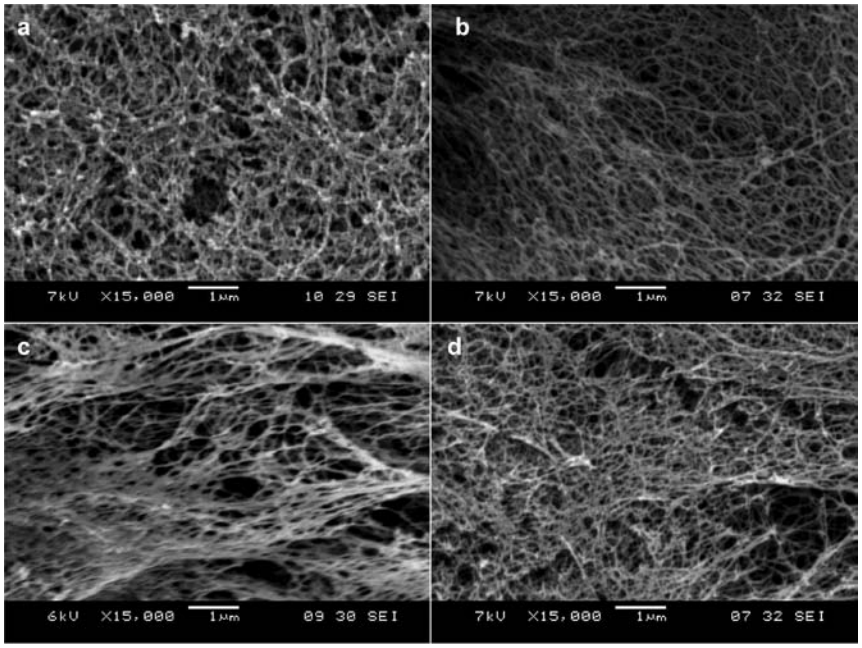

Fig. 7 SEM images of Matrigel and various designer peptide nanofiber scaffolds. a) Matrigel, b) RADA16, c) RADA16-BMHP1, d) RADA16-BMHP2 nanofiber scaffolds assembled in PBS solutions. Matrigel nanostructures are comparable in size to nanofibers found after self-assembly of the designer peptides. Clusters and aggregates of the unidentified naturally derived proteins in Matrigel (a) are absent in the pure peptide scaffolds shown in (b), (c) and (d). The interwoven nanofibers are $\sim$10 nm in diameter in each of the peptide scaffolds with $\sim$5 nm–200 nm pores. The appended functional motifs did not prevent peptide self-assembly.[17]

their microfiber and micropore size. Animal derived collagen gel and Matrigel contain other residue materials that are not always adequate for finely controlled studies. Thus, designer scaffold becomes more desirable (Fig. 8).

In order to achieve the fine-tuning and control, we have designed tailor-made peptides to suit for specific individual needs of studies or applications through appending specific active motifs onto the basic peptide, such as RADA16-I or EAK16-II.

From synthetic organic chemistry aspect, both of peptides C- or -N termini could be attached to the modified motifs. However, the functional motifs are always located on the C-termini because solid phase peptide synthesis initiates synthesis from the C-termini and proceeds toward N-terminus. The longer the peptide sequence is made, the more probable the coupling error would occur. Thus in order to avoid peptide synthesis errors, the active sequence motifs should always be at the C-terminus without exception (Fig. 6).[16–23]

Usually a spacer comprising 2-glycine residues is added to guarantee 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 (Fig. 6). Upon exposure to solution at neutral pH, the functionalized sequences self-assemble, leaving the added motifs on both sides of each

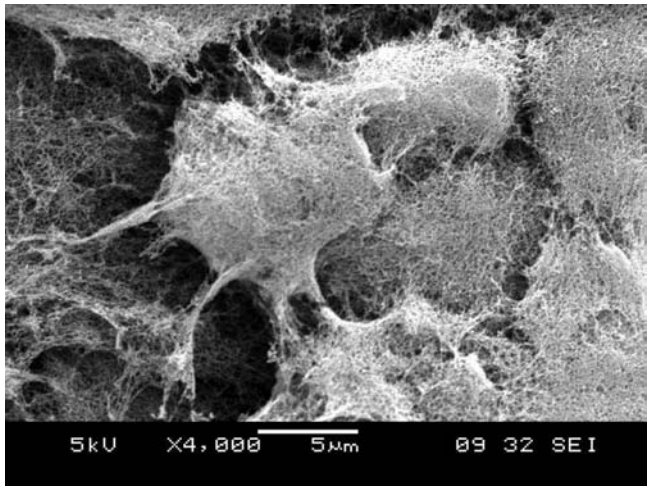

Fig. 8 SEM images of adult mouse neural stem cells (NSC) embedded in designer peptide nanofiber scaffold RADA16-BMHP1 (1% v/w) after 14 day *in vitro* cultures. Cluster of three visible mouse neural stem cells embedded in 3-D self-assembling RADA16-BMHP1. It is important to point out that the nanoscales of peptide scaffold and the extracellular matrix made by cells are indistinguishable. This is totally unlike the many processed biopolymer microfibers that are often in 10–50 µm in diameter, which is 1000–5000 bigger.[17]

nanofiber (Fig. 6). Nanofibers take part in the overall scaffold, thus providing functionalized microenvironments with specific biological stimuli (Fig. 6).

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 the study of cell interactions with their local- and micro-environments, cell migrations in 3D, 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. We showed that the addition of motifs in some cases to the self-assembling peptide RADA16-I did not significantly inhibit self-assembling properties. Furthermore, one can combine the RADA16-I nanofiber with the active designer self-assembling peptides by mixing the modified peptides. Although their nanofiber structures are indistinguishable from the RADA16-I scaffold, the appended functional motifs significantly influence 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 soluble factors. Cells reside in a 3-D environment where the extracellular matrix receptors on 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.

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 surface receptors and in most cases, larger than the cells themselves. There, cells are not in a real 3-D environment, but rather, they are on a 2-D surface wrapping around the microfiber polymers with a curvature that depends on the diameter of the polymers. It is plausible in a 2-D environment, where only one side of the cell body is in direct contact with the surface, that receptor clustering at the attachment site may be induced; on the other hand, the receptors for growth factors, cytokines, nutrients and signals may be on the other sides that directly expose to the culture media. Perhaps 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. Thus growth factors may form a gradient in 3-D nanoporous microenvironment.

In our search for additional functional motifs, we found that a class of bone marrow homing peptides BMHP is one of the most promising active motifs for stimulating adult mouse neural stem cell (NSC) adhesion and differentiation. This observation suggests a new class of designer self-assembling peptides for 3-D cell biology studies.[17]

We attached several functional motifs including cell adhesion, differentiation and bone marrow homing motifs on the C-termini. We used them to study neural stem cell, and these functionalized peptides underwent self-assembly into nanofibers structure as well. More functionalized self-assembling peptides have been shown to promote specific cellular responses and long-term cell survival, proliferation, migration, and morphological differentiation, but these peptides are expensive for most applications.[17–23]

Designer self-assembling peptide scaffolds are also shown interesting interactions with functional proteins for study of molecular releases since the release kinetics results suggested that protein diffusion through nanofiber scaffolds primarily depended on the size of proteins.[24–27]

For lipid-like peptide surfactants, some expanded to choose 2-4 amino-acid to have useful applictions. Another way is from primary sequence geometry angle to design cone-shaped, Ac-GAVILRR-NH_2, has a hydrophilic head with two positive charges and a relatively large head size and a hydrophobic tail with decreasing hydrophobicity and side-chain size with a cone shape, can self-assembly of interesting nanodonut structure.

3 Diverse uses of self-assembling peptide nanofibers

A wide range of diverse uses in various areas have been developed from these self-assemlbing peptide materials. They include 1) reparative and regenerative medicine, 2) accelerated wound healing in human clinical studies, 3) sustained molecular releases (small molecules, protein growth factors and monoclonal antibodies), 4) stabilization of deiverse membrane proteins in solution and dry surface for nanobiotechnological device fabrications, and 5) production of G protein-coupled receptors. Some of they are highlighted below.

3.1 Reparative and regenerative medicine

The self-assembling peptides are easy to use in the tissue engineering, wound healing, addressing chronic wound problems, and regenerative medicine

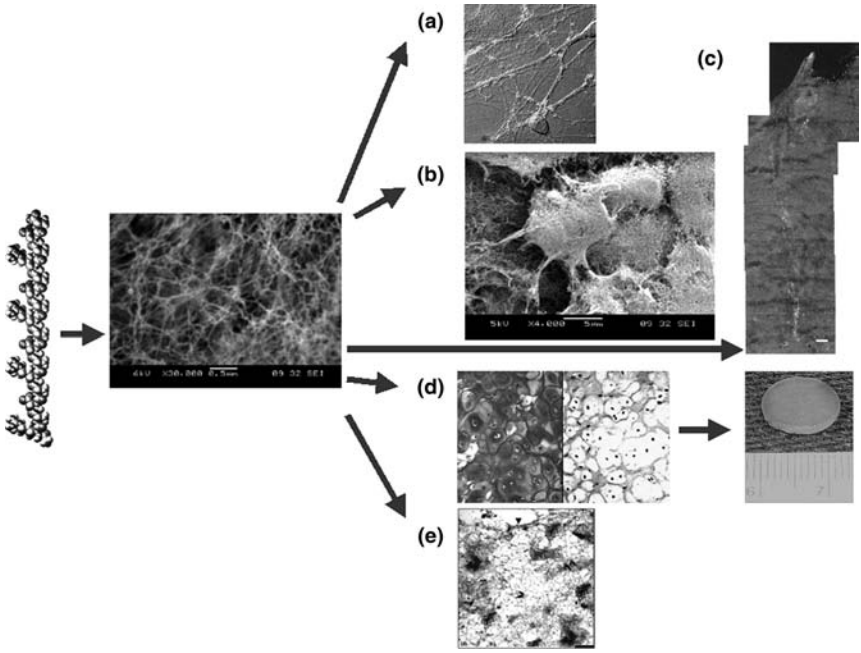

Fig. 9 From designer peptide to scaffold to tissues. **a)** Active synapses on the peptide surface. Primary rat hippocampal neurons form active synapses on peptide scaffolds. The confocal images shown bright discrete green dot labeling indicative of synaptically active membranes after incubation of neurons 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 (image courtesy of Rutledge Ellis-Behnke). The gap was sealed by the migrating cells after a few days. A great number of neurons form synapses. **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).[7]

(Fig. 9). Ellis-Behnke and colleagues used RADA16-I to repair injured rat and mouse brain structures, and their result showed the peptide scaffold hydrogel was excellent not only for axons to regenerate through the site of an acute brain injury but also to knit the injured brain tissue together seamlessly. This work represents an enabling nanobiomedical technology for tissue brain repair and restoration (Fig. 9).

Ellis-Behnke and colleagues found that the self-assembling peptide scaffolds are useful in repairing the damaged brain.[25] They also found that the peptide scaffold hydrogel instantly stopped bleeding in a few seconds during the procedure of repairing injured brain. They then expanded to study haemostasis of brain, spinal cord, femoral artery, and liver of rat.[26–28]

The early self-assembling peptide scaffold work has inspired others to expand to different kinds of tissues, organs and animals.[29–33] RADA16 functionalized with biologically active motifs also induced favourable reparative injured spinal cords.[34–35]

In order to better understand the individual molecular and material building blocks, their structures, assembly properties, dynamic behaviours and application for rapid haemostasis, we also used D-amino acids, the chiral self-assembling peptide d-EAK16 also forms 3-dimensional nanofiber scaffold. This study not only provided insights for understanding the chiral assembly properties for rapid haemostasis, but also to aid in further design of self-assembling D-form peptide scaffolds for clinical trauma emergency.[29–30]

3.2 Sustained molecular deliveries

Since the self-assembling peptides form the nanofiber scaffolds and its process is dynamic over the time, it is possible to use it for control drug delivery of molecular medicine, for small molecules and large proteins.[35–44]

Using EAK16 II, RAD16-II and RAD16-I as a model to slow release hydrophobic molecules showed that these types of self-assembling peptide scaffolds encapsulated hydrophobic drug. Various dye molecules including phenol red, bromophenol blue, 8-hydroxypyrene-1, 3, 6-trisulfonic acid trisodium salt, 1, 3, 6, 8-pyrenetetrasulfonic acid tetrasodium salt, and Coomassie Brilliant Blue G-250 through RADA16 hydrogels, providing an alternate route of controlled release of small molecules.[36] Furthermore, nanofiber encapsulated Camptothecin or ellipticine have confirmed to inhibit tumour growth.

The scaffolds have also used for sustained release of proteins including lysozyme, trypsin inhibitor, BSA, MMP-13 and monoclonal antibody IgG (Fig. 10),[37] active cytokines βFGF, TGF, VEGF and BDNF.[38] Furthermore, It can be used for sustained release from a few days to over 100 days (>3 months) when the experiments were terminated.[39] It is likely for sustained-release much longer since the content has not decrease

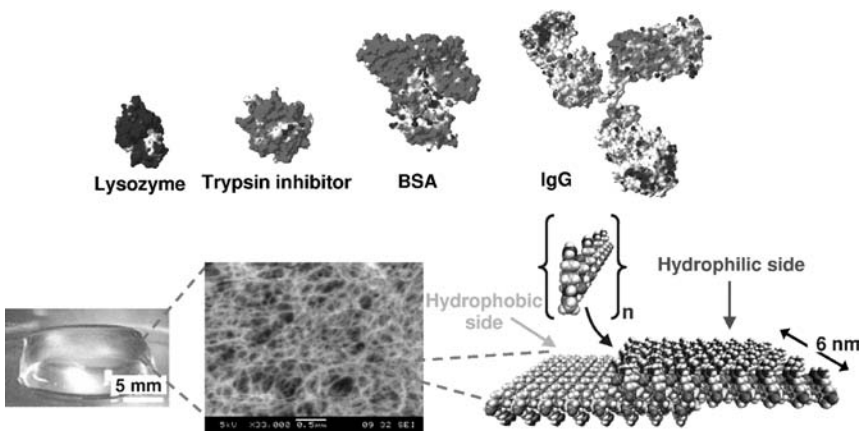

Fig. 10 Molecular representation of lysozyme, trypsin inhibitor, BSA, and IgG as well as of the ac-$(RADA)_4$-$CONH_2$ peptide monomer and of the peptide nanofiber. Color scheme for proteins and peptides: positively charged (blue), negatively charged (red), hydrophobic (light blue). Protein models were based on known crystal structures.[37] [Colour image available on-line]

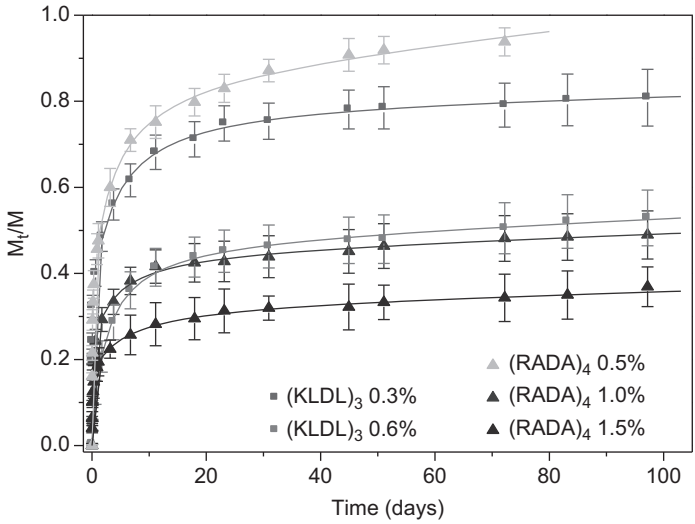

Fig. 11 The release profiles during the entire 3-month period for IgG through hydrogels of different peptides and different peptide nanofiber densities. Hydrogels consisted of the self assembling peptides (i) ac-(RADA)4-CONH2 with concentration 0.5% w/v (light blue, ▲), 1.0% w/v (blue, ▲), and 1.5% w/v (dark blue, ▲) and of (ii) ac-(KLDL)3-CONH2 with concentration 0.3% w/v (red, ■) and 0.6% w/v (magenta, ■). Release experiments were performed in PBS, pH 7.4 at room temperature. Data points represent the average of 5 samples.[39]

signficantly when the experiment results were collected (Fig. 11).[39] Future experiments will be carried out to test these ideas.

Our results not only provide evidence for long-term sustained molecular release from self-assembling peptide scaffolds, but also inspire others to design more self-assembling peptides to control molecular release for clinical applications.

3.3 Self-assembling peptides materials for human clinical studies of accelerated-wound healing

Human wound healing clinical process still seem to be similar to procedures used many years ago, use in suture during operations, try to prevent infection and other treatment measures. Accelerated-wound healing will benefit both patients and clinical workers. However, accelerated surgical and trauma wound healing especially chronical diabetic ulcer wound healing, is still problematic, the self-assembling medical technology may allieviate the problem and put to good practice.

The self-assembling peptide scaffolds have been found to be permissible for all tissue cell cultures including human cells tested, and the degradation products of the scaffolds are natural amino acids that pose no harm to human body. Furthermore a variety of stringent animal tests with rigorous controls have showed that the peptides did not elicit noticeable immune responses, nor caused measurable inflammatory reactions through injections and surgical procedures at various tissue sites. It thus encouraged people to carry out human clinical trial for accelerated-wound healing indication.

Of all subjects treated for various surgical wounds including cardiovascular surgeries for coronary artery bypass and synthetic blood vessel replacement, gastrointestinal surgery for partial hepatectomy and

gastrointestinal treatment for endoscopic excision of mucosa. Those clinical results are very encouraging and beneficial for patients. There were no observed adverse and undesirable side effects so far. This is not surprising since the designer self-assembling peptide scaffolds are totally pure synthetic amino acid based materials; there are no animal-derived impurities, no chemical and biological contaminations, no organic solvents, and no toxic compounds.

Since the successful human clinical trials, additional trials for several indications have been planned or launched for human tooth wound healing, skin wound healing from other diseases and injuries, in various parts of the world. Based on the previous successful clinical trials, it is anticipated that these clinical trials will likely be successful. It is hoped that the self-assembling peptide scaffolds will become an enabling medical technology that will truly benefit the society.

4 Surface modification self-assembling peptides

I also designed another class of self-assembling peptides that specifically assemble on surfaces to instantly change the physical, chemical and biological characteristics of surfaces.[45] In an analogy, it is like a grass lawn to cover the soil or a layer of paints to change the color and texture of surfaces. Except in this case, the coating is a nanometer layer of biological active peptides that can specifically interact with other molecules or cells. Not only we can pattern the cell for arbituary shapes, but cells can be confined in the region where they are seeded. An example of cells on the patterned surface is showing here (Fig. 12).

5 Lipid-like self-assembling peptide surfactant materials

The third classes of peptides are the lipid-like peptide surfactants, these peptides are similar to that of natural phospholipids, with tuneable hydrophobic tails to various degrees of hydrophobic amino acids such as alanine, valine or leucine, a hydrophilic head with either negatively charged aspartic and glutamic acids or positively charged histidine, lysine or arginine.[46–55] They undergo self-assembly to form well-ordered nanostructures including nanotubes, nanovesicles and micelles (Fig. 13).

5.1 A class of lipid-like self-assembling peptides

The lipid-like peptide is a new class of short peptides. Cationic, anionic, and zwitterionic peptide detergents were designed.[46–55] These lipid-like peptides are a class of molecules with properties similar to surfactants. They have hydrophilic heads comprised of 1–2 residues, and hydrophobic tails 3–6 residues long. They are about 2 nm-3 nm in length, and their ionic character and strength can be controlled by selecting appropriate amino acids or by capping the termini. Lysine or aspartic acid was used for the hydrophilic head. To control the detergent ionic nature, each peptide was capped by acetylation at the N-terminus, or selectively amidation at the C-terminus when required. Alanine, valine, leucine, and isoleucine were used for the hydrophobic tails (Figs. 13 and 14).

These lipid-like peptides behave comparably to traditional detergents, but offer several advantages over other novel detergents. Their chemical

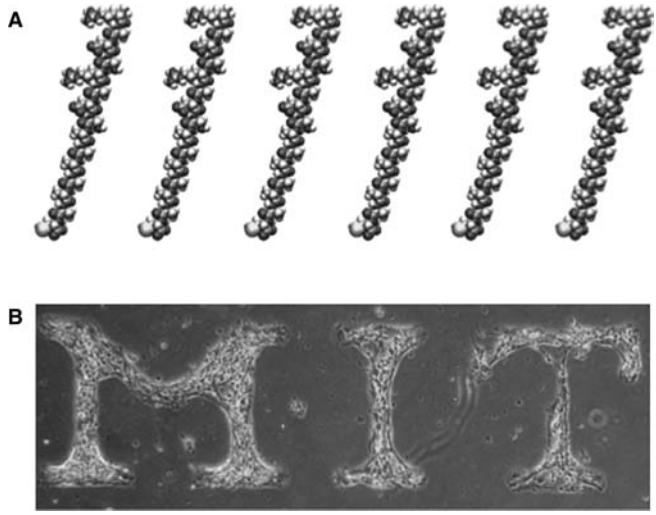

Fig. 12 Cells are patterned on the peptide coated surface. A) Molecular models of the surface self-assembling peptides. This type of peptide has three distinct segments: a biologically active segment where it interacts with other proteins and cells; a linker segment that can not only be flexible or stiff, but also sets the distance from the surface, and an anchor for covalent attachment to the surface.[45] These peptides can be used as ink for an inkjet printer to directly print on a surface, instantly creating any arbitrary pattern, as shown here. B) Mouse neural cells are seeded on the coated surface. The cells only attach where the adhesion surfaces and not the areas with non-adhesive substrate, such as oligoPEG.

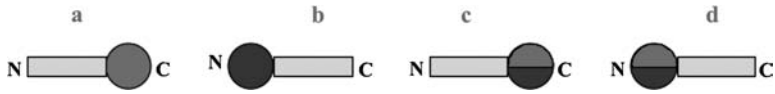

Fig. 13 The lipid-like peptides. These peptides have a hydrophilic head and a hydrophobic tail, much like lipids or detergents. They sequester their hydrophobic tail inside of micelle, vesicles or nanotube structures and their hydrophilic heads expose to water. At least three kinds molecules can be made, with $-$, $+$, $-/+$ heads and in 2 orientations.

properties are similar to commonly used detergents, they can be systematically designed and produced at high purity, and they remain stable for long periods of time.

5.2 Structure and of nanotube and nanovesicle formations

The lipid-like self-assembling peptides can form the ordered-nanotubes and nanovesicles (Figs. 15 and 16),[46–57] but the mechanism is not clear. A plausible path from the monomer state to the assemblies, two peptides form dimmer tail-to-tail packing to form a bilayer, monomeric peptides form small segments of the bilayer ring, with hydrophobic tails packing together to avoid water and hydrophilic heads exposed to water on the inner and outer portion of the tube, subsequently stack through non-covalent interactions to form longer nanotubes (Fig. 17).

I also designed with cone-shaped peptide Ac-GAVILRR-$CONH_2$ with large size amino acid arginin at the C-terminus and progressly reduce the size to glycine at the N-terminus.[51–52]

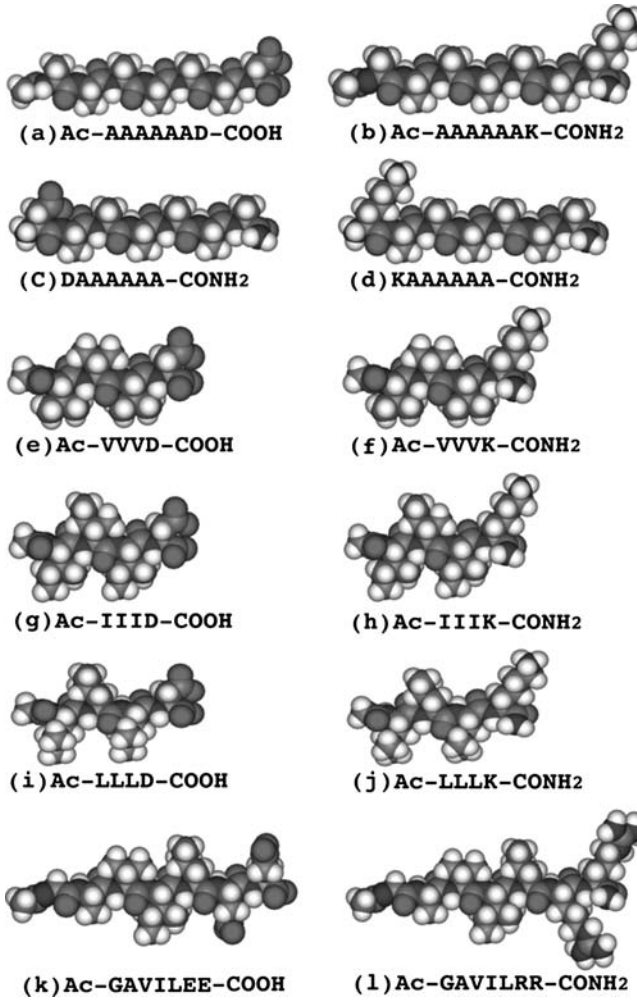

Fig. 14 Molecular models of peptide detergents at neutral pH. a) Ac-AAAAAAD-COOH. b) Ac-AAAAAAK-$CONH_2$. c) DAAAAAA-$CONH_2$. d) KAAAAAA-$CONH_2$. e) Ac-VVVD-COOH. f) Ac-VVVK-$CONH_2$. g) Ac-IIID-COOH. h) Ac-IIIK-$CONH_2$. i) Ac-LLLD-COOH. j) Ac-LLLK-$CONH_2$. k) Ac-GAVILEE, l) Ac-GAVILRR. Aspartic acid (D) is negatively charged and lysine (K) is positively charged. The hydrophobic tails of the peptide detergents consist of alanine (A), valine (V), isoleucine (I) and leucine (L). Each peptide is $\sim$2 nm–2.5 nm long, similar size to biological phospholipids. Color code: teal, carbon; red, oxygen; blue, nitrogen and white, hydrogen. [Colour image available on-line]

Charlotte A.E. Hauser and colleagues, have also discovered some ultra-small amphilic peptides as short as 3 to 7 residues that form strong hydrogels with a wide range of applications including tissue cell cultures, cosmetics, spine repair and more.[58–61] One of the peptides form very stable α-helical strucutre, one of the shortest peptide that could form helical structure.[56]

Further study of the mechanism is great importance because these lipid-like peptides have successfully stabilized diverse membrane proteins including GPCRs and used for molecular deliveries.

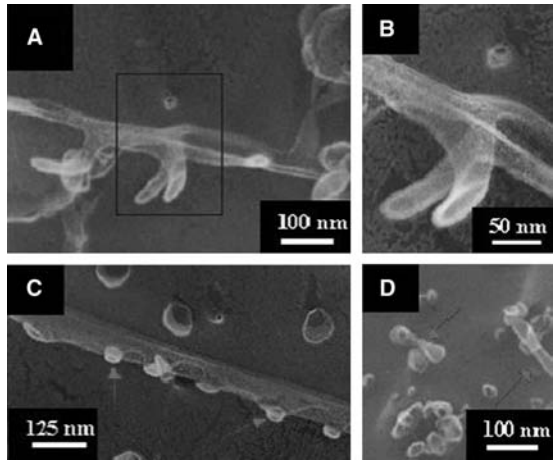

Fig. 15 High-resolution of TEM images of Ac-G_6D_2 showing different structures and dynamic behaviors of these structures. A) A pair of finger-like structure branching off from the stem. B) Enlargement of the box in (A), the detail opening structures are clearly visible. C) The openings (arrows) from the nanotube where may resulted in the growth of the finger-like structures. Some nanovesicles are also visible. D) The nanovesicles may undergo fission (arrows). (image courtesy of Dr. Steve Yang).[47]

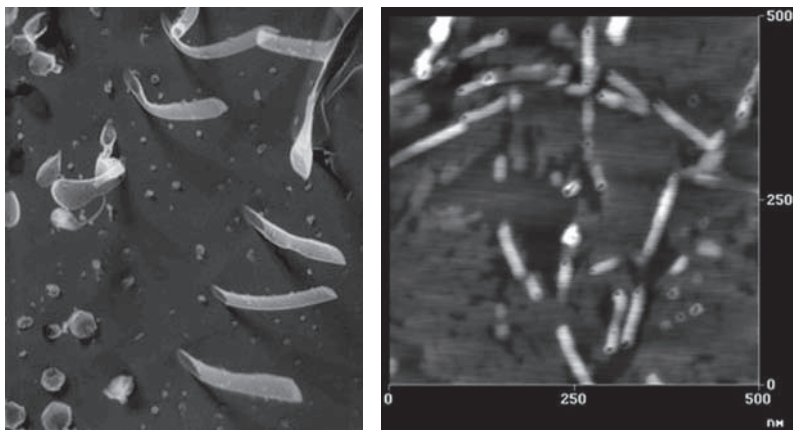

Fig. 16 (Left panel) TEM of nanotubes and nanovesicles of lipid-like peptide ac-VVVVVVD-OH in water. Micelles are also present (image courtesy of Dr. Steve Yang). (Right panel) The AFM image of nanotubes of A_6K lipid-like self-assembling peptides.[46] When the solution pH is less than the Lysine pKa 10, the peptide bears a positive charge. The openings of peptide nanotubes are clearly visible.[50] These nanotube structures can also undergo structural changes depending on various conditions, particularly pH changes, ionic strength of salts, temperature and incubation time. The other sheets like materials are likely the un-assembled peptides at the time of the image collected.

5.3 Stabilize membrane proteins

Membrane proteins play vital roles in all living systems. They involve in energy conversions, cell-cell and cell-environmental communications and sensing, specific ion channels and pumps, transporters, and all sorts of transports. Membrane proteins are also essential for our 5 senses: sight,

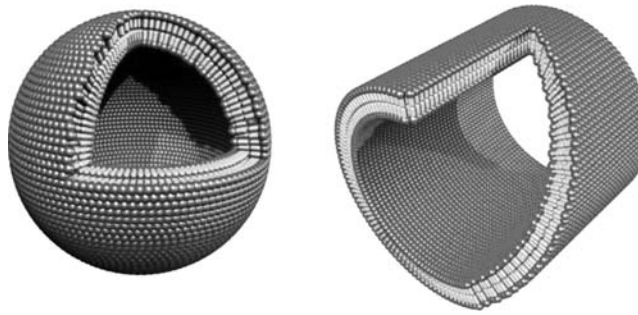

Fig. 17 Molecular modeling of cut-away structures formed from the peptides with negatively charged heads and non-polar tail. Peptide nanotube with an area sliced away. Peptide nanovesicle. Color code: red, negatively charged aspartic acid heads; green, non-polar tail. The non-polar tails are packed inside of the bilayer away from water and the aspartic acids are exposed to water, much like other surfactants. The modeled dimension is 50 nm–100 nm in diameter. [Colour image available on-line]

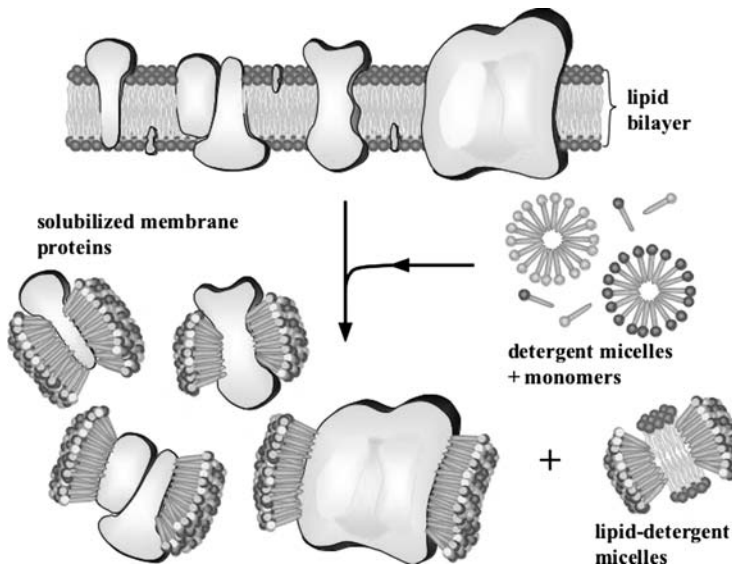

Fig. 18 A proposed scheme for how the designer lipid-like peptides stabilize membrane proteins. These simple designer self-assembling lipid-like peptides have been used to solubilize, stabilize and crystallize membrane proteins. These peptides have a hydrophilic head and a hydrophobic tail, much like 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.

hearing, smell, taste, touch and temperature sensing; and G-protein coupled receptors (GPCRs) are crucial in learning, memory, stem cell renewal and differentiation, body-plan development, the immune system, aging and more. However, our understanding of their structures and function falls far behind than that of soluble proteins (Fig. 18).

For past a few years, our teams gradually overcome some of the obstacles of the membrane proteins production and purification. We solubilized and

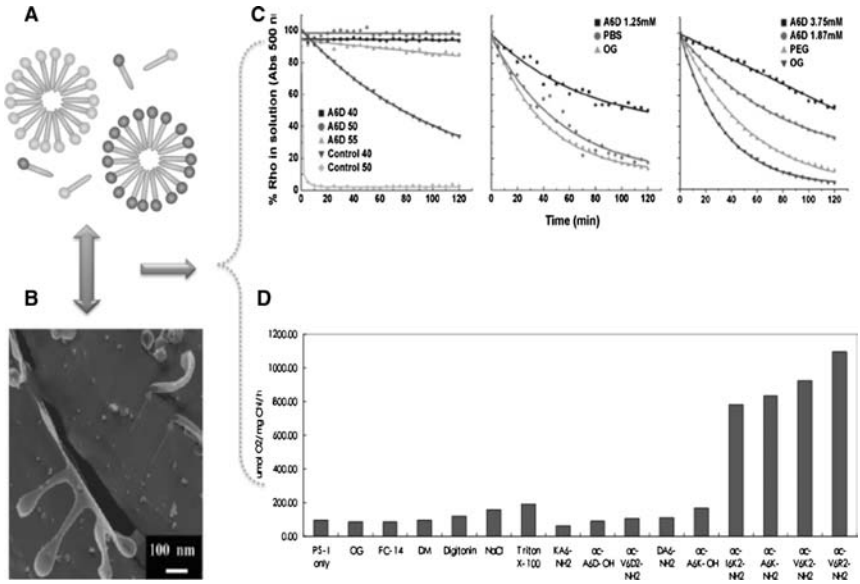

Fig. 19 Designer self-assembling peptides stabilize membrane proteins. (A) A proposed model peptide surfactants; (B) Quick-freeze/deep-etch TEM image of peptide surfactant (V_6D) dissolved in water; Stability kinetics of rhodopsin (Rho) in different surfactants; (C) Kinetics of bovine rhodopsin under different conditions. Stability of rhodopsin in the absence of OG at different temperatures. Half-life of rhodopsin was as follows: not available in 2.5 mM A_6D at 40 °C, 50 °C, and 55 °C (Left); (D) Decay of A500 in delipidated rhodopsin in the absence of OG. Half-life of rhodopsin was as follows: 122 min in 1.25 mM A_6D, 47 min in PBS, and 27 min 1% OG (Middle); Stability of delipidated rhodopsin at 40 °C (Right);[65] Lipid-like peptides stabilize functional photosystem I.[64]

stabilized several classes of membrane proteins using the lipid-like peptides, including *E.coli* Glycerol-3-phosphate dehydrogenase,[62] the multi-domain protein complex Photosystem-I (PSI) on surface in dry form[63] and in aqueous solution,[64] G protein-coupled receptor bovine rhodopsin (Fig. 19)[65] and many olfactory receptors.[66–68]

5.4 Lipid-like peptides used in cell-free productions of high yield membrane proteins

Cell-free production of proteins is a robust method and capable for molecular fabrications quickly, often in a few hours. It can easily be industrially standardized with rigorous quality controls. The technology is simple and versatile, unskilled person can be trained in a few hours to perform the task. Various functional proteins including membrane proteins, particularly difficult G-protein coupled receptors and other 7-transmembrane proteins (7TM), have been made for a variety of studies and for a wide range of applications.

Recently, cell-free protein productions have become cost effective and competitive. Grams amount of functional proteins and materials can be made in a few hours instead of weeks. Cell-free system can be integrated into other devices because it's simplicity and robustness.

Selecting the right surfactant is thus crucial because bottlenecks in elucidating the structure and function of membrane proteins are the difficulty

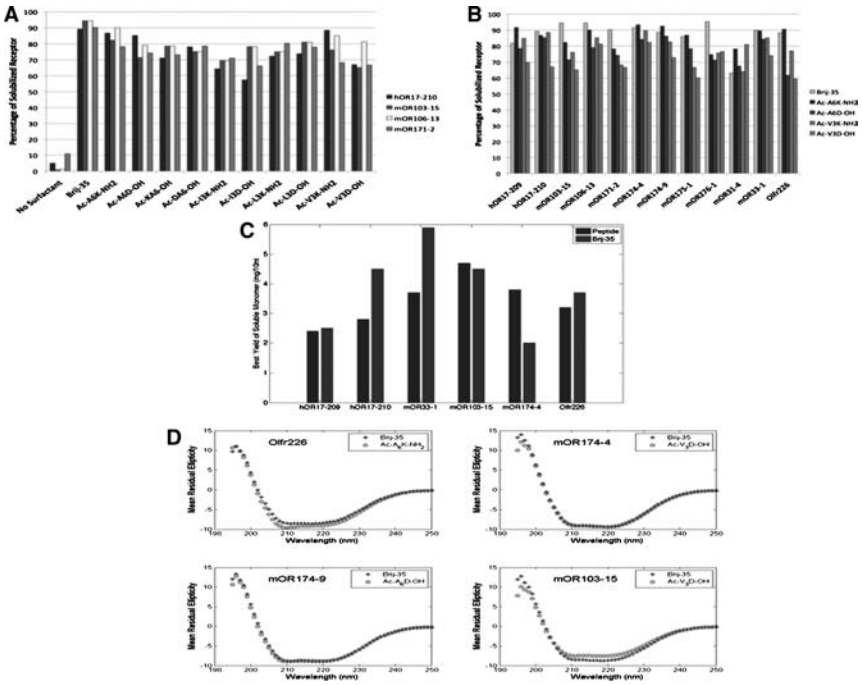

Fig. 20 Designer lipid-like peptides are used in the cell-free systems. Tens of functional olfactory receptors purification and secondary structure have been achieved. Olfactory receptors are solubility in Brij-35 and peptide detergents. Each receptor was expressed in the presence of Brij-35 or a peptide detergent using a commercial *E. coli* cell-free expression system (Qiagen, RiNA and Invitrogen). (A) The presence of a detergent was necessary to solubilize the olfactory receptors, and all of the peptide detergents were able to solubilize four unique receptors; (B) The detergent peptides and Brij-35 were able to solubilize similar fractions of protein. Peptides that were positively charged or had longer tails tended to solubilize higher fractions of receptors; (C) Detergent peptides can yield milligram quantities of solubilized olfactory receptors, and the maximum yield of the monomeric form of all tested olfactory receptors expected in a 10 ml reaction. Only results from the most effective detergent peptide are shown; (D) CD spectra of Brij-35 and peptide detergent-produced olfactory receptors to Olfr226, mOR174-4, mOR174-9 and mOR103-15 (Images courtesy of Karolina Corin).[66–67]

of producing large quantities of functional receptors. Various self-assembling peptide surfactants in commercial *E.coli* cell-free systems can rapidly produce milligram quantities of soluble G-protein coupled receptors (GPCRs) that include the human formyl peptide receptor (FPR), human trace amine-associated receptor (TAAR), vomeronasal type 1 receptor 1 (VNR), and other olfactory receptors.[66–67]

Furthermore, using short, designer lipid-like peptides as surfactants, we not only produced 12 unique mammalian olfactory receptors, but also solubilized and stabilized to maintain their structure and function (Fig. 20).[67–68] These simple and inexpensive lipid-like peptide surfactants will likely make significant contributions to facilitate the rapid production of GPCRs, and perhaps other membrane.

6 Summary

Since the unexpected discovery of the self-assembling peptide EAK16-II in yeast Zuotin, we have come a long way, from initial surprises, puzzlement,[1–3]

no understanding at all to, in outline, not only gradually understand the design principals at the molecular level, the molecular and fine material structures, interactions of the peptides, the dynamic self-assembly behaviours, but also how we can further improve their designs. From there, we not only subsequently expanded designer materials using 20 natural L-amino acids or some non-natural D-amino acids, but also we proceeded to optimize their sequence for 3D tissue cell cutlures,[18–24] delivering bioactive therapeutics such as drugs and growth factors and antibodies.[36–40] Recent advances in functionalization have also led to the development of better synthetic tissue culture bioactive scaffolds that promote cell proliferation, migration and differentiation for regenerative medicine. Furthermore, these self-assembling peptides have become an enabling medical technology[24–30,69–80] that will find a wide ranges of uses in surgery, emergency care, accelerated wound healing and more.

In science there are numerous examples of curiosity-driven research and unintentional discoveries that eventually lead to technological breakthroughs and new economic development. It is extremely difficult to imagine not to pursue the curiosity-driven research and explorations. Curiosity-driven research not only provide new insights into mysteries of nature and generate new knowledge, but it also can translate the knowledge into new enabling technology that will eventually be developed knowledge-based economy.

There are few examples: X-ray, the structure of DNA double helix, DNA-RNA and RNA-RNA hybridizations, reverse transcription, RNA splicing, RNA as enzymes, telomeres, Natural Killer cells (NK cells), programmed cell death, microRNA, RNA interference, S-layer proteins, Kuru disease, carbon 60, carbon nanotubes, carbon graphene and the indispensible worldwide web -- www. The discovery of the self-assembling peptide is another good example that an unexpected curiosity-driven discovery led to the development of an enabling medical technology that will benefit the society. The recent example of successful clinical trials of the self-assembling peptide scaffolds for accelerating wound healings and regenerative medicine provide a glimpse of what is coming for wide spread uses of chiral self-assembling peptides. Therefore curiosity-driven research must be strongly encouraged and fully supported, despite the current emphasis of application-driven research.

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