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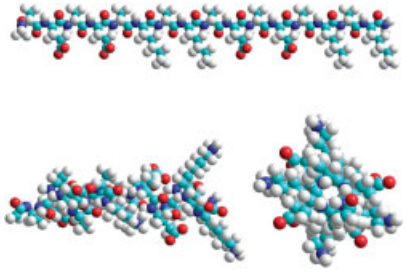
Full Paper

The use of D-amino acids to form a designer self-assembling peptide nanofiber is reported. The chiral D-peptide is shown to give a mirror-image circular dichroism spectrum of its L-analogue. The D-peptide nanofibers can form scaffolds for possible tissue growth and are shown to be highly resistant to protease degradation, which may facilitate their use in biotechnology, nanobiotechnology, tissue repair, and tissue regeneration as well as other medical technologies.



Self-Organization of a Chiral D-EAK16 Designer Peptide into a 3D Nanofiber Scaffold

Z. Luo, X. Zhao, S. Zhang*

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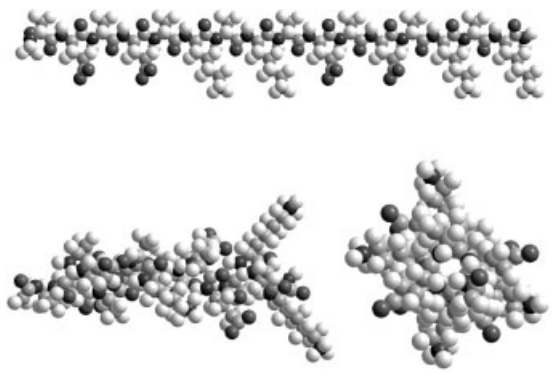
Self-Organization of a Chiral D-EAK16 Designer Peptide into a 3D Nanofiber Scaffold

Zhongli Luo, Xiaojun Zhao, Shuguang Zhang*

Self-assembling peptide nanofiber scaffolds are an excellent material for applications such as tissue repair, tissue regeneration, instant stopping of bleeding, and slow drug release. We report a new self-assembling peptide D-EAK16 consisting purely of D-amino acids. D-EAK16 and L-EAK16 display mirror-image CD spectra at 20 °C. Like L-EAK16, D-EAK16 self-assembles into nanofibers, thus demonstrating that chiral self-assembling peptide nanofiber scaffolds can be made from both L- and D-amino acids. We also show that D-peptide nanofibers are resistant to natural proteases and may thus be useful in biotechnology, nanobiotechnology, tissue repair and tissue regeneration as well as other medical applications.



Introduction

The design of molecular biological materials requires detailed structural knowledge to build advanced materials and complex systems.[1–3] We previously studied a self-assembling peptide EAK16-II, which consists of 16 natural L-amino acids. The EAK16-II peptide was originally found as a segment in a yeast protein Zuotin, which was characterized by its ability to preferentially bind to the left-handed Z-DNA.[4] These self-assembling peptides have alternating hydrophobic, e.g., alanine, valine, leucine, isoleucine, and phenylalanine, and hydrophilic sides, which include positively charged lysine, arginine, histidine, and negatively charged aspartic acids and glutamic acids. Their hydrophilic sides have been classified into several charged modulus: modulus I, modulus II, modulus III, and modulus IV are $-+-+-+-+$, $--++--++$, $---+++$, and $----++++$, respectively.[5] These designer self-assembling peptides have been found to be very useful in diverse areas including culturing functional cartilage *in vitro*,[6] repairing the optical nerve system in a hamster brain,[7] instantly stopping bleeding in animals,[8] repairing heart myocardial infarction,[9] and three dimensional (3D) tissue cell culture systems.[10–12]

In nature, there are several examples of synthetic peptides with D-amino acids, they include hormones bradykinin,[13] oxytocin,[14] and angiotensin[15] and peptide antibiotics enantio-enniatin B.[16–19] But they do not undergo molecular self-assembly to form defined and well-ordered scaffolds.

Here we report a self-assembling peptide D-EAK16 made of only D-amino acids. In addition to forming nanofibers it can also self-organize into 3D nanofiber scaffolds, like its L-counterpart. The peptide can form a beta-sheet molecular structure with D-chirality to give the mirror image of the circular dichroism (CD) spectrum of its L-peptide counterpart at 20 °C but is less stable at 80 °C. The structure is sensitive to pH conditions, which is similar to the L-form peptide reported in a previously study. Since the peptides

Z. Luo, X. Zhao, S. Zhang
West China Hospital, Laboratory for Nanobiomedical Technology, Sichuan University, Chengdu, Sichuan 610065, China
E-mail: Shuguang@mit.edu

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made of only D-amino acids are resistant to natural proteases, the D-peptide-containing materials and devices would be more durable. Thus, it will be likely broadly useful in biotechnology, nanobiotechnology, and medicine.

Experimental Part

Design and Purification of the D-EAK16 Peptide

The peptide D-EAK16, of sequence Ac–A*E*A*E*A*K*A*K*A*E*A*E*A*K*A*K*–$CONH_2$ (where * denotes a D amino acid), was custom-synthesized by solid-phase peptide synthesis (Figure 1). The peptide synthesis used standard *N-tert*-butyoxycarbonyl (*t*-Boc) chemistry in cycles using *N*-methylpyrrolidone.[20] Acetyl and amino groups protected the *N*-terminus and C-terminus of the peptide, respectively. The crude peptides were purified by HPLC and characterized by mass spectrometry.[21] The degree of purity of D-EAK16 was 98.11% (L-EAK16[4] was 95.02%). The lyophilized white powder was stored at 4 °C. Solutions of the peptides were prepared at concentrations of 1–10 $mg \cdot mL^{-1}$ (0.1–1.0%) in water (18 $M\Omega \cdot cm$, Millipore Milli-Q system) and stored at 4 °C before use.

Scanning Electron Microscopy (SEM) Sample Preparation

The peptide samples were prepared as follows: 2–10 mg of the powder of D-EAK16 peptide (10 $mg \cdot mL^{-1}$) was added to 200–1 000 μL of phosphate-buffered saline (PBS, pH = 7.2), cell culture media DMEM, or RAMP1640. The samples for SEM (JSM-5900, JEOL, Japan) were prepared by first incubating the membranes in 5% glutaraldehyde at 4 °C for 30 min and then dehydrating them sequentially with 20, 50, 70, 90, and 100% ethanol and liquid CO_2. The specimens were examined using SEM from 5 000× to 50 000× magnifications.

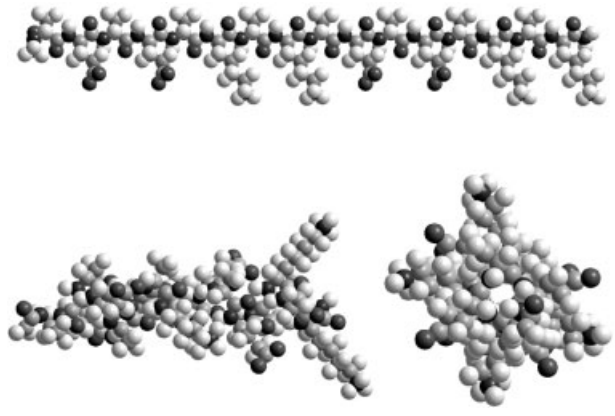

Figure 1. Molecular models of D-EAK16. D-EAK16 in the extended β-strand (N → C) (alanine on *N*-terminus is to left and lysine on C-terminus is to the right); α-helical model, horizontal (left) and α-helical model viewing from the center of axis (right) (alanine on *N*-terminus is on top and lysine on C-terminus is on the bottom). Color code: hydrogen = white, carbon = cyan, oxygen = red, and nitrogen = blue.

Atomic Force Microscopy (AFM) Sample Preparation

Aliquots of 5–10 μL were removed from the peptide solution (0.1–2 $mg \cdot mL^{-1}$) at various times after preparation and were deposited onto a freshly cleaved mica surface. To optimize the amount of peptide adsorbed, each aliquot was left on mica for 30–60 s and then washed with 100 μL of deionized water at least 3 times. The mica surface with the adsorbed peptide was then air-dried and imaged immediately. The images were obtained by scanning the mica surface in air using AFM (Hitachi model SPM400, Japan) operating in the tapping mode. When imaging the soft biopolymers using AFM at high-resolution, it was important to minimize the tip tapping force. Thus, soft silicon cantilevers were chosen (SI-DF2000, K-A102001604, Japan) with a spring constant of 1–5 $N \cdot m^{-1}$ and a tip radius of curvature of 5–10 nm. AFM scans were taken at 512 × 512 pixels resolution and produced topographic images of the samples, in which the brightness of features increases as a function of height. Typical scanning parameters were as follows: Scanner *X/Y/Z*: 53.00/53.00/4.19 $nm \cdot N^{-1}$, lever $k_z/k_t/f_0$: 20.00 $N \cdot m^{-1}$/100.00 $N \cdot m^{-1}$/150.00 kHz, length/tip 200.0/10 μm, data type: topography (servo), area/speed 100–1000 nm/1.0–1.5 Hz, amp. ref.: −0.1 to −0.3, vib. voltage: 1.0–2.0 V, bias: 0.000 V, integral and proportional gains 0.03–0.3 and 0.1–0.5, respectively.

Circular Dichroism (CD) Spectroscopy

The peptide samples were prepared by diluting the stock solution (100×10^{-6} M). After preparation the peptide solution was immediately placed into a quartz cuvette with a path length of 2 mm. CD spectra were recorded (AVIV Model-400, software version is v3.02a) several times after preparation. The thermal behavior of the peptide sample was determined by increasing the temperature and equilibrating the samples in increments of 2 °C through 30 s temperature equilibrium. Samples were scanned from 250 to 190 nm. The bandwidth was 1 nm and the averaging time was 1 s. The settling time was 0.333 s and three scans were averaged for each sample. CD spectra were converted to mean residue ellipticity.

Protease Treatment

Stock solutions of enzymes containing trypsin, pepsin, protease K, and pronase were diluted and mixed with an equal volume of solution to a final concentration of 250 $\mu g \cdot mL^{-1}$. The peptide samples were prepared at 331.6 $\mu g \cdot mL^{-1}$ (PBS, pH = 7.2). The enzymes and peptides were mixed with an equal volume to obtain a solution of enzymes and the peptide samples with final concentrations of 125 and 165.8 $\mu g \cdot mL^{-1}$, respectively. All mixed samples were incubated at 37 °C overnight (≈15 h). After the incubation, 5–10 μL of solution was placed on the mica to determine whether the fibrils were degraded. The mica samples were washed in pure water for 20–30 s and air-dried in a covered dish at

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room temperature for 3 h. The peptide nanofibers were observed by AFM scanning of the mica surface that contained the samples.

Peptide Scaffold Preparations

The peptide scaffold samples were prepared as follows: 5–10 μL of the stock solution of D-EAK16 peptide 10 mg · mL^{-1} (1%) was added to 0.5–1.0 mL PBS (150×10^{-3} M NaCl/10×10^{-3} M sodium phosphate, pH = 7.4) with 0.00001% Congo red in a glass slide.

Results and Discussion

Structural Properties of D-EAK16

From a previous study, L-EAK16 is known to form an exceedingly stable β-sheet structure.[4] We were interested in what structure the chiral D-EAK16 peptide would adopt. Would it have an inverted CD spectrum? CD spectroscopy revealed that the D-EAK16 indeed has an inverted beta-sheet spectrum with a maximum ellipticity at 218 nm and a minimum ellipticity at 193 nm (Figure 2A). The CD profiles for D-EAK16 and L-EAK16[4] are almost perfect mirror images, which suggests that they are chiral to each other (Figure 2A). However, when heating both peptides to high temperature, above 80 °C, followed by cooling to 25 °C, L-EAK16 retained the beta-sheet CD spectrum, but the D-EAK16 CD spectrum changed to more of a non-typical beta spectrum with a broadened absorption from 208–222 nm (Figure 2B). This observation suggests that the beta-sheet structure of D-EAK16 is less stable than that of L-EAK16.

Scaffold Hydrogel Formation

We previously demonstrated that L-EAK16 formed a scaffold hydrogel with extremely high water content, namely, >99% water.[4] Because such scaffold hydrogels are very useful for a number of applications, including the study of cells in a more realistic 3D microenvironment,[11,12] we investigated if our scaffolds could be used in a similar manner.

D-EAK16 (10 mg · mL^{-1}) indeed forms a scaffold hydrogel with >99% water content (Figure 3). The scaffold hydrogel not only formed in PBS but also in the tissue cell culture media DMEM and RAMP1640. When the scaffold hydrogel was stained with Congo red, it became easily visible by the naked eye (Figure 3). This observation is consistent with previous report of L-EAK16[4] and other self-assembling peptide EFK8[5] and KLD12.[6]

Molecular modeling shows that the D-EAK16 peptide has eight moderate hydrophobic D-alanine residues on one side and ionic self-complementary hydrophilic pairs of four positively charged D-lysines and four negatively charged D-glutamic acids on the other side. These four complementary ionic bonds are likely important to stabilize the β-sheets, which increases the stability of the nanofiber structure and facilitates the scaffold self-assembly process.

We showed that the designer self-assembling peptides form stable β-sheets and a scaffold hydrogel. They can be made from L-amino acids and from D-amino acids. This suggests that peptides made from homo-chiral amino acids do not hinder scaffold hydrogel formation. However, it remains to be seen if it holds true for the mixed chiral forms, namely, both L- and D-amino acids in a single peptide. Some studies are under way.

Formation of Well-Ordered Nanofibers and Influence of pH

We have previously shown that L-EAK16 forms well-ordered nanofibers with a defined structure.[4,22] Since its sequence is identical to L-EAK16, except with its D-chirality, D-EAK16 was investigated for its ability to also form nanofibers. AFM examination of D-EAK16 revealed that D-EAK16 did indeed form ordered nanofibers (Figure 4) that ranged from a few hundred nanometers to a few micrometers when the peptide was incubated overnight in PBS (pH = 7.4).

However, when at pH = 1.1, D-EAK16 formed vesicles or globules (Figure 5A). At pH = 12, D-EAK16 mostly formed nanoaggregates (Figure 5B). These observations are consistent with the secondary structure observed by CD. When at neutral pH, D-EAK16 forms a stable β-sheet, but at pH = 1 and 12, D-EAK16 is converted into from a β-sheet to an α-helix structure.[23] Similar observation of pH sensitivity for L-EAK16 was also reported.[24]

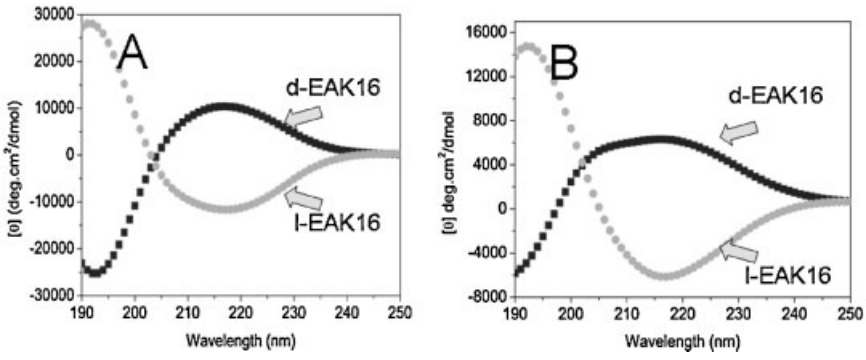

Figure 2. CD spectra of peptides D-EAK16 and L-EAK16 in water. The *x*-axis is wavelength in nm; the *y*-axis is expressed as mole residue ellipticity [θ]. The mirror image of D-EAK16 and L-EAK16 reflects the chirality. A) The peptides were incubated in water at 20 °C. Note the mirror image of the β-sheet spectrum. B) The peptides were incubated in water at 80 °C. Note the different spectra: the beta-sheet spectrum of L-EAK16 and helical-like spectrum of D-EAK16.

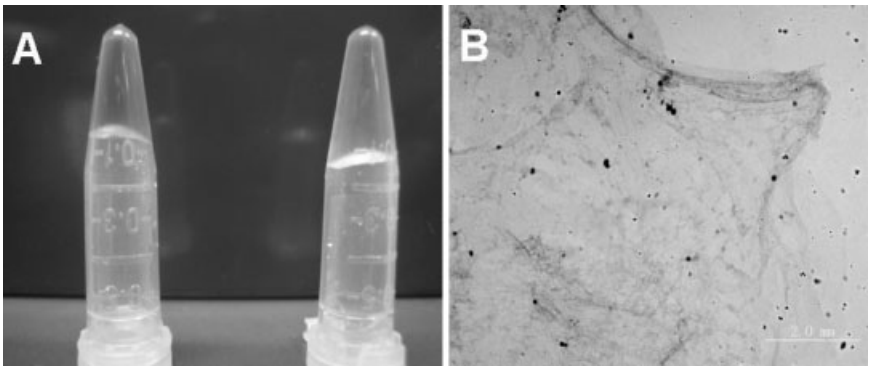

Figure 3. Photographs of macroscopic membrane hydrogels of D-EAK16. The peptide concentration is 10 mg · mL^{-1} (1% peptide, 99.0% water). A) The left inverse micro-tube is a self-assembly with the cell culture medium (DMEM) and the right is D-EAK16 in the PBS solution. B) A colorless membranous structure was formed in PBS and transferred to a glass slide and can then be seen by the naked eye, it is isobuoyant. The image is stained bright red with Congo red.

The Peptide Scaffold Formation

It has previously been shown that peptides can form a 3D matrix, which is very useful to culture a number of tissue cells *in vitro*.[12,25,26] Since D-EAK16 can also form a nanofiber scaffold, it could be very useful for the study of tissue cells in 3D cultures without the concern of scaffold degradation.[10–12] They could be useful for the culture of tissue cells since peptides and proteins made of D-amino acids are resistant to degradation by natural proteases, thus the material is more durable. We examined the fine structure of D-EAK16 in the cell culture media DMEM or RAMP1640 using SEM (Figure 6).

SEM images revealed well-ordered nanofibers and nanopores from 0.5% D-EAK16 peptides, which is similar to that observed for the fine structure of L-EAK16.[4,11,22] The D-EAK16 self-assembles into a 3D scaffold that exhibits a nanometer-scale structure (Figure 6). The nanostructure of D-EAK16 is very important to surround cells in a true 3D scaffold where cells can then establish their own microenvironments quickly. It seems likely that D-EAK16 will be useful not only in basic 3D cell biology, tumor biology, and neurobiology, but also in cell-based high-content drug screening for pharmaceutical and biotechnology industries and beyond.

Protease Degradation

After the formation of D-EAK16 nanofibers, they were investigated for their ability to resist protease degradation, including against trypsin, α-chymotrypsin, papain, protease K, and pronase. It is assumed that the D-form peptide bonds cannot be cleaved by proteases made of L-amino acids, thus D-EAK16 may resist natural enzyme degradation.

We carried out experiments of protease treatments and observed that the nanofibers could withstand protease degradation. The D-EAK16 nanofibers in enzymatic solution appeared to bundle much more tightly than in PBS solution. The nanofibers seemed to further aggregate to each other in the solution of proteases and formed an interconnected network with pores in the range of ≈50 nm (Figure 5C). This is consistent with a similar report of a peptide made partly of D-amino acids that is highly resistant against proteolytic degradation in dilute human serum and in lysosomal preparation.[27] Our observations suggest that the D-EAK16 nanofibers are not significantly degraded by the proteases under our current experimental conditions.

Thermal Behavior of D-EAK16

Although the D-EAK16 is very stable at physiological temperature 37 °C, it becomes less stable than that of

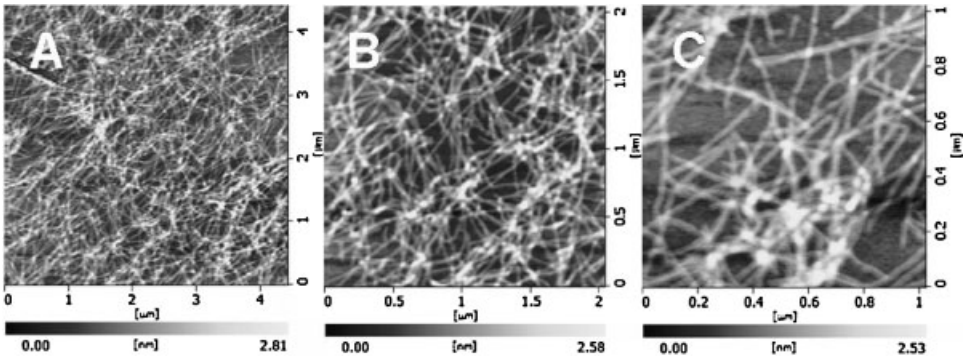

Figure 4. AFM images of D-EAK16 self-assembled into nanofibers (2 mg · mL^{-1} or 0.2% w/v). The images were serially collected at increasing magnifications. A) Low-resolution image 4.3 μm^2, B) higher resolution, 2 μm^2, and C) highest resolution 1 μm^2. The well-ordered nanofibers and their pores are clearly visible. The diameter of the nanofibers is about to 10 nm. These images are similar those for L-EAK16 reported previously.

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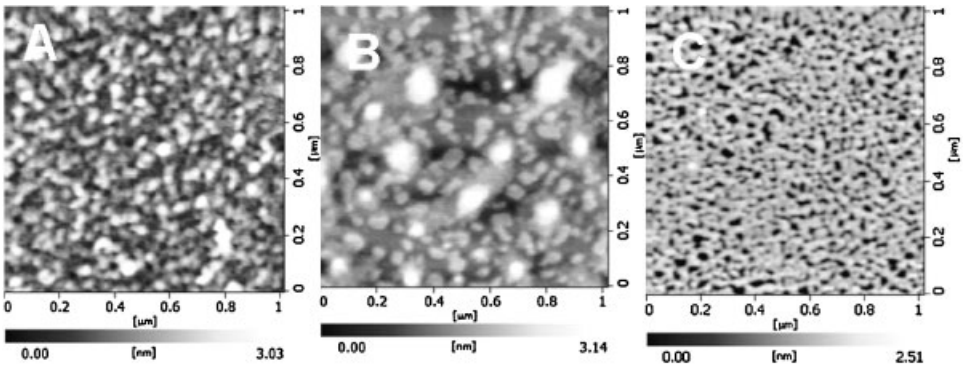

Figure 5. AFM images of D-EAK16 in acidic medium and upon degradation by enzymes (2 mg · mL^{-1} or 0.2% w/v). The D-EAK at A) pH = 1, B) pH = 12.8. When the peptide solution is close to pH = 1 or pH = 12, the peptides aggregated to form globular structures. C) D-EAK16 was incubated with proteases. The proteases including trypsin, pepsin, protease K, and pronase were diluted and mixed with an equal volume of solution to a final concentration of protease and peptide of 125 μg · mL^{-1} each.

L-EAK16 when incubated at a temperature higher than 80 °C. In the CD experiments, when the D-EAK16 solution was heated to 80 °C (Figure 2B), the CD spectrum changed towards a more A-helical-like structure, whereas the L-EAK16 was not significantly changed even heating at 80 °C.[23] A systematic and detailed study of this thermal behavior has been reported elsewhere.[23]

The Chirality of Self-Assembly

Molecular self-assembly is the spontaneous organization of molecules under near thermodynamic equilibrium conditions into structurally well-defined and stable arrangements through weak non-covalent interactions. Such interactions typically include hydrogen bonds, electrostatic attractions, and van der Waals interactions. Although these bonds are relatively insignificant in isolation, when combined together as a whole, they govern the structural conformation of all biological macromolecules and influence their interaction with other molecules. Many factors can influence the behavior of peptide self-assembly, including i) the amino acid sequence, ii) the concentration of the peptide, iii) the molecular size of the peptide, iv) the 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) and urea.[4,22,28–36] We now show that the homogeneous chirality of the amino acids is important to self-assembly. Detailed molecular behavior studies of heterogeneous peptides that contain both D- and L-amino acids will be reported elsewhere.

D-EAK16 and L-EAK16 are enantiomers. D-EAK16 and L-EAK16 peptides have mirror image CD spectra at 20 °C (Figure 2A). D-EAK16 and L-EAK16 consist of identical chiral amino acids, either D- or L-isomers, respectively, they also both contain two-unit repeats of alternating hydrophilic and hydrophobic residues. Our observations suggest that the homochirality of the amino acids has little influence on the weak non-covalent interactions of the ionic complementary residues for the peptide structures. Although they have a chiral structure, the self-assembly of the nanofibers is insensitive to the chirality alone. When the self-assembling peptides are made of either homo-L- or D-amino acids, the nanofibers are indistinguishable under our experimental conditions (Figure 4, Figure 6), except for their chirality. It is not

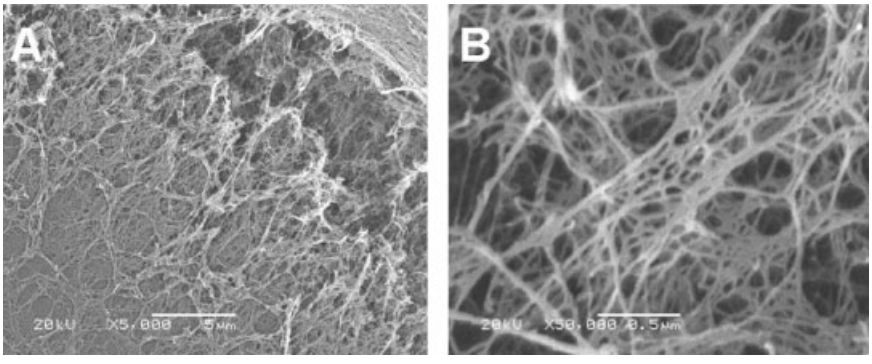

Figure 6. SEM serial photograph of D-EAK16. The diameter of the nanofibers is about to 10 nm, and the pores are about 20–500 nm. A) 5 000× and B) 50 000× magnifications. The nanofiber structure of D-EAK16 is similar to that of L-EAK16. The diameter of the nanofibers of L-EAK16 is ≈10 nm, and the pores of the scaffold are about 20–500 nm as reported previously.[4,11,25]

surprising that regardless of chirality the molecular self-assembly takes place through a balance of weak non-covalent interactions that include hydrogen bonding, electrostatic, hydrophobic, and van der Waals forces, and water-mediated interactions that result in the formation of well-defined structures.[30,37,38]

Our results obtained from studies of temperature, pH, concentration of salt, and the denaturation agents showed that the D-EAK16 and L-EAK16 behaves similarly in many ways except at temperatures greater than 80 °C with prolonged incubation.[23] It appears that at 80 °C or higher, the D-EAK16 is less stable than the L-EAK16, perhaps the weak interactions in the D-amino acids are slightly less stable than those in the L-amino acids. More studies are required to clarify the observations.

Implications for the D-Amino Acid Systems

There is some discussion about using D-peptides for chaperone systems[39] and for inducing protective immunity.[40] Shai and colleagues reported that a synthetic D-amino acid peptide that corresponded to the *N*-terminal sequence of HIV-1 gp41 (D-WT) of HIV-1 associates with its enantiomeric wild-type fusion (WT) peptide in the membrane and inhibits cell fusion mediated by the HIV-1 envelope glycoprotein.[41] Kim and colleagues[42] also reported the fusing domain *per se* as a target for drug development, in which the D-peptide inhibitors are likely to be useful for development and identification of a new class of orally bioavailable anti-HIV drugs.[42]

A pharmacologically important GsMTx4 peptide made of only D-amino acids demonstrated that modulation of membrane proteins by amphipathic peptide-mechano-pharmacology involves not only the protein itself but also the surrounding lipids with surprising efficiency and important therapeutic implications.[43]

Here we suggest that, like its chiral counterpart, D-EAK16 may be useful as a more durable designer extracellular matrix (ECM) because it can not only self-assemble into ordered nanofiber scaffolds but these scaffolds can also be used as a micro-environment for 3D culture of diverse cells.[44] They may also be useful as scaffolds to fill anatomical defects for tissue repair and tissue engineering.[44] These scaffolds may not only be designed to provide the desired mechanical properties necessary to support cells, but it also may be bioengineered to contain and then slowly release growth factors, small molecule drugs, peptide and protein therapeutics, as well as RNAi and DNA genes to stimulate tissue regeneration.

Our study lies at the interface between molecular biology, biochemistry, polymer science, and materials science. The study of molecular self-assembling peptides made of L- and D-amino acids will likely continue to reveal nature's secrets. We anticipate more surprises from this new class of self-assembling peptide system in the future.

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