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Peptide self-assembly in functional polymer science and engineering

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Abstract

Biological self-assembly systems lie at the interface between molecular biology, chemistry, polymer science and engineering. The key elements in molecular self-assembly are chemical complementarity and structural compatibility. Several types of self-assembling peptides have been engineered. Type I peptides undergo intermolecular self-assembly, type II peptides undergo self-assembly and disassembly, i.e. intermolecular and intramolecular self-assembly under the influence of various conditions. Type III peptides undergo self-assembly on to surfaces. These self-assembling peptide systems are simple, versatile and easy to modify and to produce. These systems represent a significant advancement in the molecular engineering of protein fragments for diverse technological innovations.

Keywords: Cell attachment; Cell pattern formation; Ionic complementarity; Molecular switch; Neurite outgrowth; Peptide matrices; Structural transformation; Surface engineering

1. Introduction

Molecular self-assembly, by definition, is the spontaneous organization of molecules under thermodynamic equilibrium conditions into structurally well-defined arrangements due to noncovalent interactions. These molecules undergo self-association forming hierarchical structures without external instruction. Molecular self-assembly is ubiquitous in nature and has recently emerged as a new strategy in chemical synthesis, polymer science and engineering [1]. Molecular self-assembly is the basis of polymers at the early stage before polymerization.

Proteins and protein fragments have not been considered to be useful materials for traditional engineering science, although their potential has been recognized. Several recent discoveries and rapid developments in biotechnology, however, have rekindled the field of biological materials engineering [2–7]. In the last few years, considerable advances have been made in the use of peptides as building blocks to produce biological materials for a wide range of applications. A new class of oligopeptide based biological materials was serendipitously discovered from the self-assembly of ionic self-complementary oligopeptides [7–11]. A number of

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self-assembling peptides have been designed and analyzed (Table 1). These systematic analyses provided insight into the chemical and structural principles of peptide self-assembly. This class of biomaterials has considerable potential in a number of applications, including platforms or scaffolding for tissue engineering [9,12], drug delivery of protein and peptide medicine, as well as biological surface engineering [13]. These peptides are short, simple to design, extremely versatile and easy to synthesize. Three types of self-assembling peptides have been systematically studied thus far. It is believed additional different types will be discovered and developed over time.

Type I peptides form beta-sheet structures in aqueous solution because they contain two distinct surfaces, one hydrophilic, the other hydrophobic. The unique structural feature of these peptides is that they form complementary ionic bonds with regular repeats on the hydrophilic surface. The complementary ionic sides have been classified into several moduli, i.e. modulus I, II, III, IV, etc., 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, molecules of modulus I have − + − + − + − +, modulus II, − − + + − − + +, modulus, IV− − − − + + + +. Upon the addition of monovalent alkaline cations or the introduction of the peptide solutions into physiological media, these oligopeptides spontaneously assemble to form macroscopic structures that can be fabricated into various geometric shapes [12,14]. Scanning EM reveals that the matrices are made of interwoven filaments that are about 10–20 nm in diameter and pores about 50–100 nm in diameter [7,9,14]. A variety of mammalian cell types have been shown to attach to the peptide matrices. These peptide materials can also have various biological properties by incorporating different biological ligands [9,12].

Type II peptides were discovered while analyzing a family of modulus IV peptides. One of the peptides with 16 amino acids, (DAR16-IV), has a beta-sheet circular dichroism spectrum at ambient temperature but showed an abrupt transition at high temperatures to a stable alpha-helical spectrum without a detectable random coil intermediate [11]. On cooling, it retained the alpha-helical form and took several weeks at room temperature to partially return to the beta-sheet. Similar structural transformations were observed by changing the pH. Several members of the type II peptides showed similar structural changes. This suggests that secondary structures of some sequences, especially segments flanked by clusters of negative charges on the N-terminus and positive charges on the C-terminus, may undergo drastic conformational transformations under the appropriate conditions. These findings may provide insight into protein–protein interactions during protein folding and the pathogenesis of some protein conformational diseases, such as scrapie or Alzheimer's disease.

Furthermore, type III peptides have also been developed. These form monolayers on surfaces for specific cell pattern formation or to interact with other molecules. These oligopeptides have three distinct features. The terminal ligand segment was modified to incorporate a variety of functions for recognition by other molecules or cells. Since the peptide has two asymmetric N- and C-termini, the ligand can be located at either the N- or C-terminus of the peptides depending on how it is recognized by other biological moieties. Specific ligand information is usually available in the literature from extensive mutational and structural analyses of protein–protein or ligand–receptor interactions. The second feature is the central linker where a variable spacer is used that allows for freedom of interactions at a specified distance away from the surface. The third feature is in the anchor where a chemical group on the peptide can react with the surface to form a covalent bond. This

Table 1
Self-assembling oligopeptides studied[a]

Name	Sequence (n → c)	Modulus	Structure
	+ − + − + − + −		
RADA16-I	n-R A D A R A D A R A D A R A D A-c	I	β
	+ − + − + − + −		
RGDA16-I	n-R A D A R G D A R A D A R G D A-c	I	r.c.
	+ − + −		
RADA8-I	n-R A D A R A D A-c	I	r.c.
	+ + − − + + − −		
RAD16-II	n-R A R A D A D A R A R A D A D A-c	II	β
	+ + − −		
RAD8-II	n-R A R A D A D A-c	II	r.c.
	− + − + − + − +		
EAKA16-I	n-A E A K A E A K A E A K A E A K-c	I	β
	− + − +		
EAKA8-I	n-A E A K A E A K-c	I	r.c.
	+ − + − + − + −		
RAEA16-I	n-R A E A R A E A R A E A R A E A-c	I	β
	+ − + −		
RAEA8-I	n-R A E A R A E A-c	I	r.c.
	+ − + − + − + −		
KADA16-I	n-K A D A K A D A K A D A K A D A-c	I	β
	+ − + −		
KADA8-I	n-K A D A K A D A-c	I	r.c.
	− − + + − − + +		
EAH16 − II	n-A E A E A H A H A E A E A H A H-c	II	β
	− − + +		
EAH8-II	n-A E A E A H A H-c	II	r.c.
	− − + + − − + +		
EFK16-II	n-F E F E F K F K F E F E F K F K-c	II	β
	− + − +		
EFK8-II	n-F E F K F E F K-c	I	ND
	− − + + − − + +		
ELK16-II	n-L E L E L K L K L E L E L K L K-c	II	ND
	− − + +		
ELK8-II	n-L E L E L K L K-c	II	r.c.
	− − + + − − + +		
EAK16-II	n-A E A E A K A K A E A E A K A K-c	II	β
	− − − − + +		
EAK12	n-A E A E A E A E A K A K-c	IV/II	α/β
	− − + +		
EAK8-II	n-A E A E A K A K-c	II	r.c.
	+ + + + − − − −		
KAE16-IV	n-K A K A K A K A E A E A E A E A-c	IV	β
	− − − − + + + +		
EAK16-IV	n-A E A E A E A E A K A K A K A K-c	IV	β
	+ + + + − − − −		
RAD16-IV	n-R A R A R A R A D A D A D A D A-c	IV	β
	− − − − + + + +		
DAR16-IV	n-A D A D A D A D A R A R A R A R-c	IV	α/β
	− − − − + + + +		
DAR16-I[b]	n-D A D A D A D A R A R A R A R A-c	IV	α/β
	− − − − + + + +		
DAR32-IV	n-(A D A D A D A D A R A R A R A R)-c	IV	α/β
	+ − + − + + + + − + − + + + +		
EHK16	n-H E H E H K H K H E H E H K H K-c	N/A	r.c.
	+ − + − + + + +		
EHK8-I	n-H E H E H K H K-c	N/A	r.c.
	− − − − − − − − − −		
VE20[b]	n-V E V E V E V E V E V E V E V E V E-c	N/A	β (NaCl)
	+ + + + + + + + + +		
RF20[b]	n-R F R F R F R F R F R F R F R F R F-c	N/A	β (NaCl)

[a] β, β-sheet; α, α-helix; r.c., random coil; N/A not applicable; ND, not determined.
[b] Both VE20 and RF20 are in β-sheet form when they are incubated in solution containing NaCl. They do not self-assemble to form macroscopic matrices.

biological surface engineering technique will provide tools to study cell–cell communication and cell behaviors [13].

2. Type I self-assembling peptides

2.1. Molecular modeling and engineering

We designed many type I self-assembling peptides for various studies. One of the ionic self-complementary peptides, AEAEAKAK-AEAEAKAK (EAK16-II, A, alanine, E, glutamate, and K, lysine), was originally found as a repeated segment in a yeast protein [15]. Its molecular structure and proposed complementary ionic pairings between positively charged lysines and negatively charged glutamates in an overlap arrangement are illustrated in Fig. 1. This structure represents an example of this class of self-assembling beta-sheet peptides that undergo spontaneously association under physiological conditions [7–9,14]. If the charged residues are substituted, i. e., the positive charged lysines are replaced by positively charged arginines and the negatively charged glutamates are replaced by negatively charged aspartates (Fig. 1A), there are essentially no drastic effects on the self-assembly process. However, if the positively charged resides, Lys and Arg are replaced by negatively charged residues, Asp and Glu, the peptide can no longer undergo self-assembly to form macroscopic materials, although they can still form beta-sheet structures in the presence of salt. This observation is consistent with previous reports that when the peptides are incubated in the presence of salt for extended time periods or

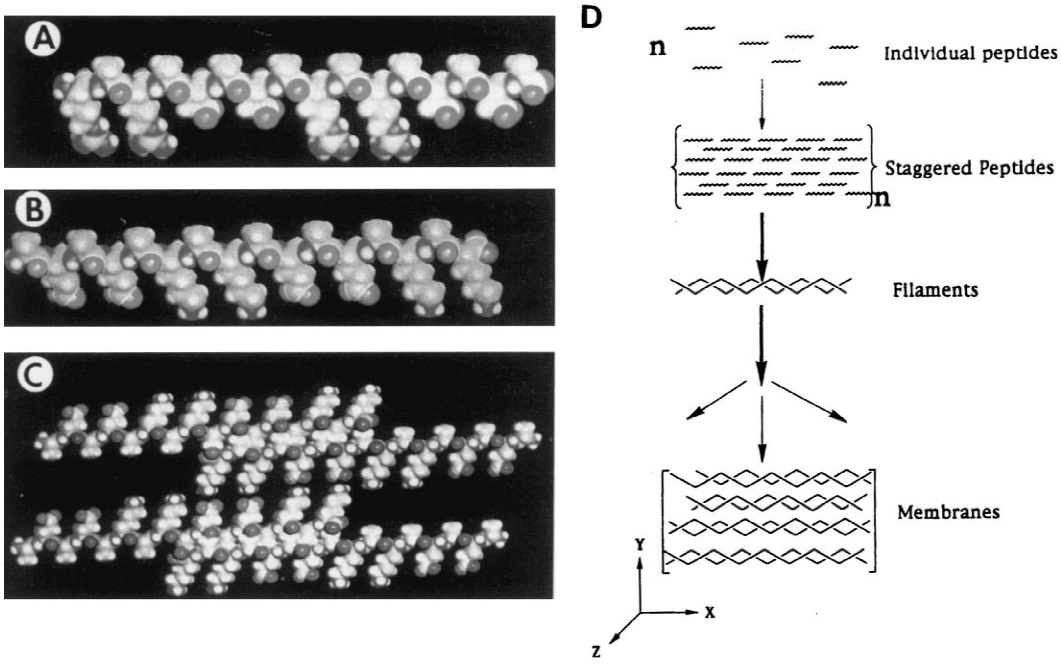

Fig. 1. Molecular models of the extended beta-strand structures of individual molecules are shown for RAD16 (A) and EAK16 (B). The distance between the charged side chains along the backbone is approximately 6.8 Å; the methyl groups of alanines are found on one side of the sheet and the charged residues are on the other side. Conventional beta-sheet hydrogen bond formation between the oxygens and hydrogens on the nitrogens of the peptide backbones are perpendicular to the page. (C) A proposed staggered assembly of molecular models for EAK16. The complementary ionic bonds and hydrophobic alanines are shown. Although an anti-parallel beta-sheet is illustrated, a parallel beta-sheet model is also possible. (D) A proposed model of sequential events which could lead to assembly of macroscopic matrices. One of the possible pathways of matrix formation. X, Y, and Z indicate three dimensions of the materials. Geometric shapes other than membrane can also be produced as suggested by the diverging thin arrows (see Fig. 2).

change of pH, these peptides can also form high molecular weight structures [16–19]. If the alanines are changed to more hydrophobic residues, such as Leu, Ile, Phe or Tyr, the molecules have a greater tendency to self-assemble and form peptide matrices with enhanced strength [14].

2.2. Peptide materials structure

When the peptides are exposed to salt solution, by delivering it into salt solution through different processing apparatus, peptides readily formed matrices that can be fabricated in various geometric forms. A close examination of these peptide materials using scanning electron microscope (SEM) revealed that the materials were made of interwoven fibers. These fibers have relatively regular fiber diameter and enclosure. Many peptide materials that are capable of matrix-formation have been examined and they all have similar appearances [7,9,14]. Fig. 2 presents examples of macroscopic peptide material and their microscopic structure at high resolution.

2.3. Mechanical properties of oligopeptide materials

The mechanical properties for one peptide material, EFK8, has recently been examined [14]. This peptide material showed some interesting mechanical properties. Its fiber density increases as the concentration of the peptide increases but not the fiber diameter. Furthermore the mechanical strength of peptide materials is proportional to the concentration of the peptide with a Young's modulus about 2 kPa and 15 kPa at 0.3% and 1% in water, respectively [14]. In the same manner, several peptides with Leu or Ile substitutions showed similar properties [unpublished results]. Interestingly, when polypeptides have only the negatively or positively charged residues with alternating Val, Leu, Phe or Tyr, they did not form visible materials [16–19]. Therefore, not only the hydrophobic residues are important, but both positive and negative charges on the same peptides are essential for peptide material formation. It should be pointed out that the peptide materials had no detectable swelling property when the material was delivered into a saline solution. This is likely due to the high water content of the material, in which $>99\%$ of the material is water. This is a very important factor if the materials are to be further developed as the scaffold for tissue engineering and tissue repair. This unique property of this material removes the chance of there being an unregulated expansion of the scaffold that could lead to adverse physiological effects on neighboring tissues.

2.4. Peptide scaffold support cell attachment and extensive neurite outgrowth

The peptide self-assembly phenomenon was a serendipitous observation during an experiment when the cytotoxicity for tissue cells was examined using the peptides. The cells exhibited a robust growth and no cytotoxicity was observed. However, a thin layer of material that was attached with the cells was observed under a phase contrast microscope. This thin layer of material occurred only in the duplicated cell culture dishes where the EAK16-II peptides were added. Whereas in other dishes where different peptides including EAK8-II (A single unit of the EAK repeat, see Table 1) were used, this phenomenon was not observed. A number of mammalian cells have been tested and all have been found to be able to form stable attachments with the peptide materials (Table 2) [9]. Several peptide materials have been tested for their ability to support cell proliferation and differentiation. These results suggested that the peptide materials can not only support various types of cell attachments, but can also allow the attached cells to proliferate and differentiate. For example, as rat PC12 cells on peptide matrices were exposed to NGF, they underwent differentiation and exhibited extensive neurite

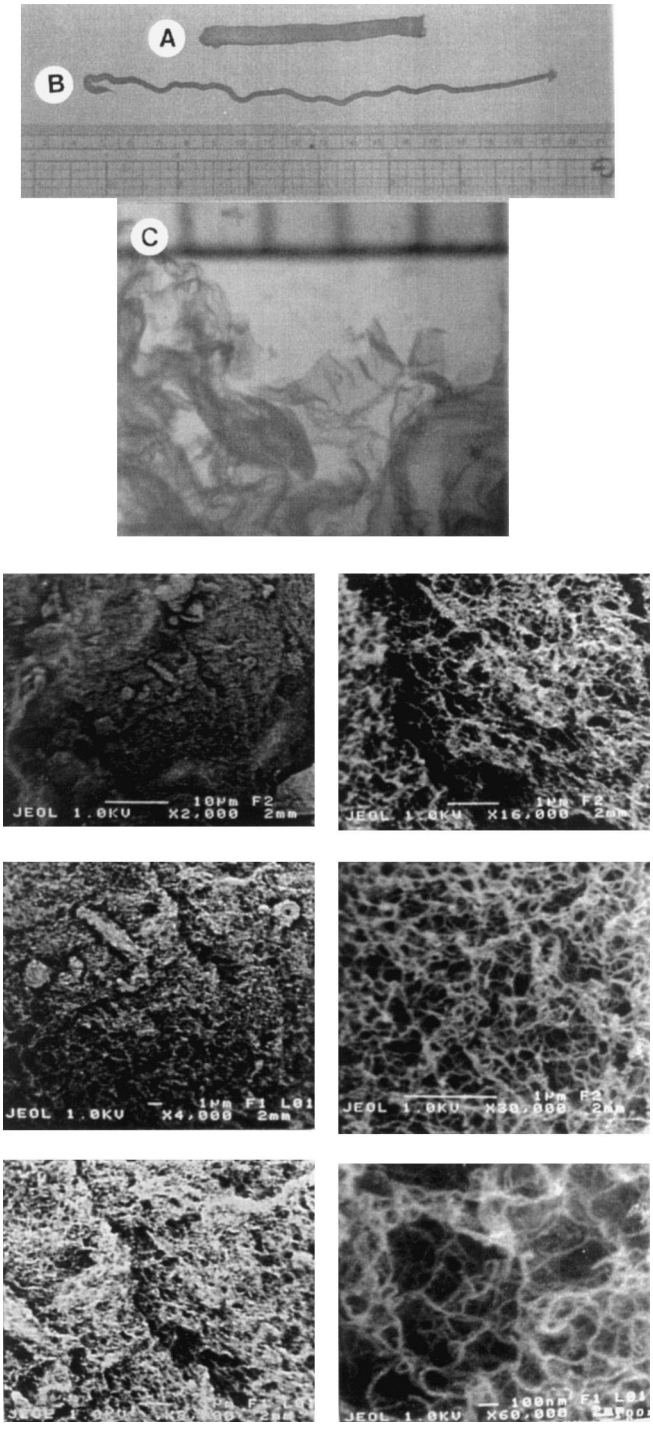

Fig. 2. Photographs of biological materials from the self-assembling peptides of RAD16-II. (A) The peptide material is fabricated as a tape with 5 mm in width, 0.2 mm in thickness and 8 cm in length. (B) A peptide matrix is fabricated as a thread with a diameter about 2 mm through a syringe. Materials of such thread with a length of greater than 20 cm has been produced from 1 ml of peptide solution. (C) The peptide matrix was fabricated as membrane, each scale is 1 mm. (D) The SEM structure of RADA16-II. The material is self-assembled from individual interwoven fibers. The diameter of the fiber is about 10–20 nm and the enclosures are about 50–100 nm. The scale bar is shown in each frame: (a) 10 mm, (b–e) 1 mm, and (f) 0.1 mm.

Table 2
Cells tested for attachment to the oligopeptide EAK16 and RAD16 matrices

Cell type[a]	Cell line
Mouse fibroblast	NIH-3T3
Chicken embryo fibroblast	CEF
Chinese hamster ovary	CHO
Human cervical carcinoma	Hela
Human osteosarcoma	MG63
Human hepatocellular carcinoma	HepG2
Hamster pancrease	HIT-T15
Human embryonic kidney	HEK293
Human neuroblastoma[c]	SH-SY5Y
Rat pheochromocytoma	PC12
Bovine aortic endothelial cells[b]	
Mouse cerebellum granule cells	
Mouse and rat Hippocampal Cells	
Human Foreskin Fibroblast	
Human Epidermal Keratinocytes	

[a] Various cell types attached to the oligopeptide matrices. Visual assessment of cell attachment was performed using phase contrast microscopy for over a period of two weeks.
[b] Refers to cells derived from primary cultures.
[c] Refers to neuronal cells.

outgrowth [12]. In addition, when primary mouse neuron cells were allowed to attach the peptide materials, the neuron cells projected lengthy axons that followed the surface contours of the self-assembled peptide materials (Fig. 3).

3. Type II self-assembling and disassembling peptides

3.1. Structural transformation of peptides from a β-sheet to an α-helix

Most peptides studied so far are very stable at various conditions. However, the results from DAR16-IV and several derivatives were a surprise. When the peptide powder was dissolved in water and measured immediately by CD, it exhibited an alpha-helical structure. However, after being stored at 4°C for a few days, it formed a stable beta-sheet structure. Upon heating at an elevated temperature, 50°C or higher, the beta-sheet abruptly transformed into an alpha-helix again. Interestingly, when standing at 23°C for several weeks, it slowly changed

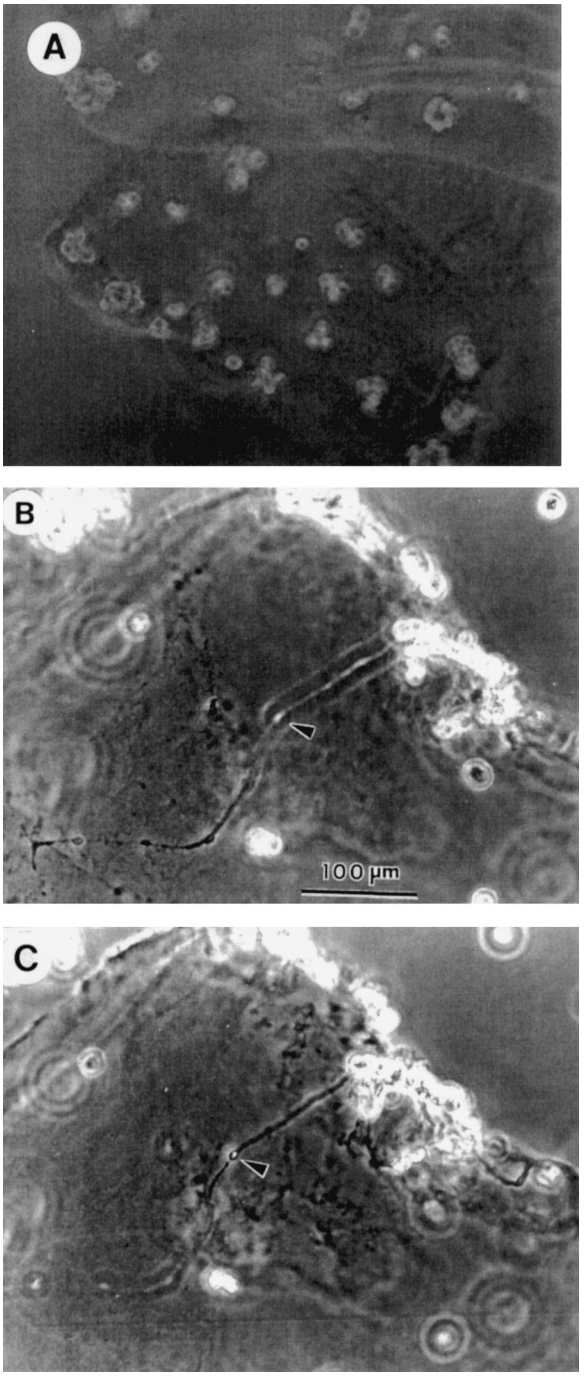

Fig. 3. The cell attachment and differentiation on the peptide material. (A) Human bone cells on the peptide materials. (B and C) Mouse neuron cells project axons on the peptide material over 0.4 mm in a few days. Because the materials do not have a flat surface, the axons on the materials is not always in focus, again, suggesting that they follow the surface contours of the peptide materials. The arrows point out the varicosity at the identical position.

back to a beta-sheet structure. It showed a considerable hysteresis (Fig. 4). The sequence of DAR16-IV has a cluster of negatively charged glutamate residues close to N-terminus and a cluster of positively charged Arg residues near C-terminus. It is well known that all alpha-helices have a helical dipole moment with partial negative C-terminus toward partial positive N-terminus [20]. Because of the unique sequence of DAR16-IV, its side chain charges balance the helical dipole moment, therefore favoring helical structure formation. However, in the special case of RAD16-IV and its derivatives, they also have alternating hydrophilic and hydrophobic residues as well ionic self-complementarity, which have been previously characterized to form stable beta-sheets. Thus, the behavior of this type of molecules is likely to be more complex and dynamic than others. Several RAD16-IV derivative peptides with such charge distributions have been designed and studied. These observations confirmed the initial findings [Altman, et al, submitted]. Recently, a yeast alpha-agglutinin protein with the sequence of EYELENAKFFK has been found to undergo conformational changes from a beta-sheet structure to an atypical helical structure at high temperature and changes of pH (Zhao, personal communication). It would be interesting to see if more such examples could be found in other proteins. This structural dynamic behavior is quite surprising, but it forces us to reconsider the problem of protein folding and protein–protein interactions. It is believed that most secondary structures in proteins are stable once they are formed. However, proteins frequently undergo catalysis, transport, interaction with other substances and involved in a wide range of interactions. It is reasonable to speculate that protein secondary structures are not static, rather, they can adopt many conformations to accommodate their biological functions.

From a polymer and materials science and engineering point of view, these structural transformations can be viewed as possible molecular switches that can be regulated by changing temperature or pH. The molecular length accompanying the structural change is approximately 2 fold so that one form could be viewed as on and the other off.

4. Type III surface self-assembling peptides

4.1. Biological surface engineering

We recently developed Type III peptides for biological surface engineering. We are interested in modifying surfaces to probe detailed molecular and cellular phenomena in a well-controlled manner. These peptides have three distinctive features: a ligand, a linker, and an anchor. All three groups can be tailored for a specific purpose. Since the motif chosen (Arg-Ala-Asp-Ser)n, or written as (RADS)n has high specificity for integrin receptors, the peptide can serve as a ligand for attaching cells to surfaces (Fig. 5). This simple system using self-assembling peptides and other substances to modify surfaces is an emerging technology that will have far reaching implications in both biomedical engineering and biology. We are interested in developing this technology further for various studies. For example, we can design various patterns to address specific questions in cell biology on how communities of cells communicate through one or two cell connections (Fig. 6). Application of external stimuli, e.g. calcium, potassium, hormones, growth factors, cytotoxic substances or electric impulses to one community of cells through micro-manipulation will allow the study of responses from the other community of cells through the messenger cells. This biological surface engineering technology using self-assembling peptides may also be useful in biomedical research and clinical applications as a new detection technique. For example, we can design a specific ligand as a ‘molecular hook’ that can interact with specific molecules on cancer cell surfaces in high affinity so as to anchor cancer cells on the surfaces. This type of diagnostic device may be manufac-

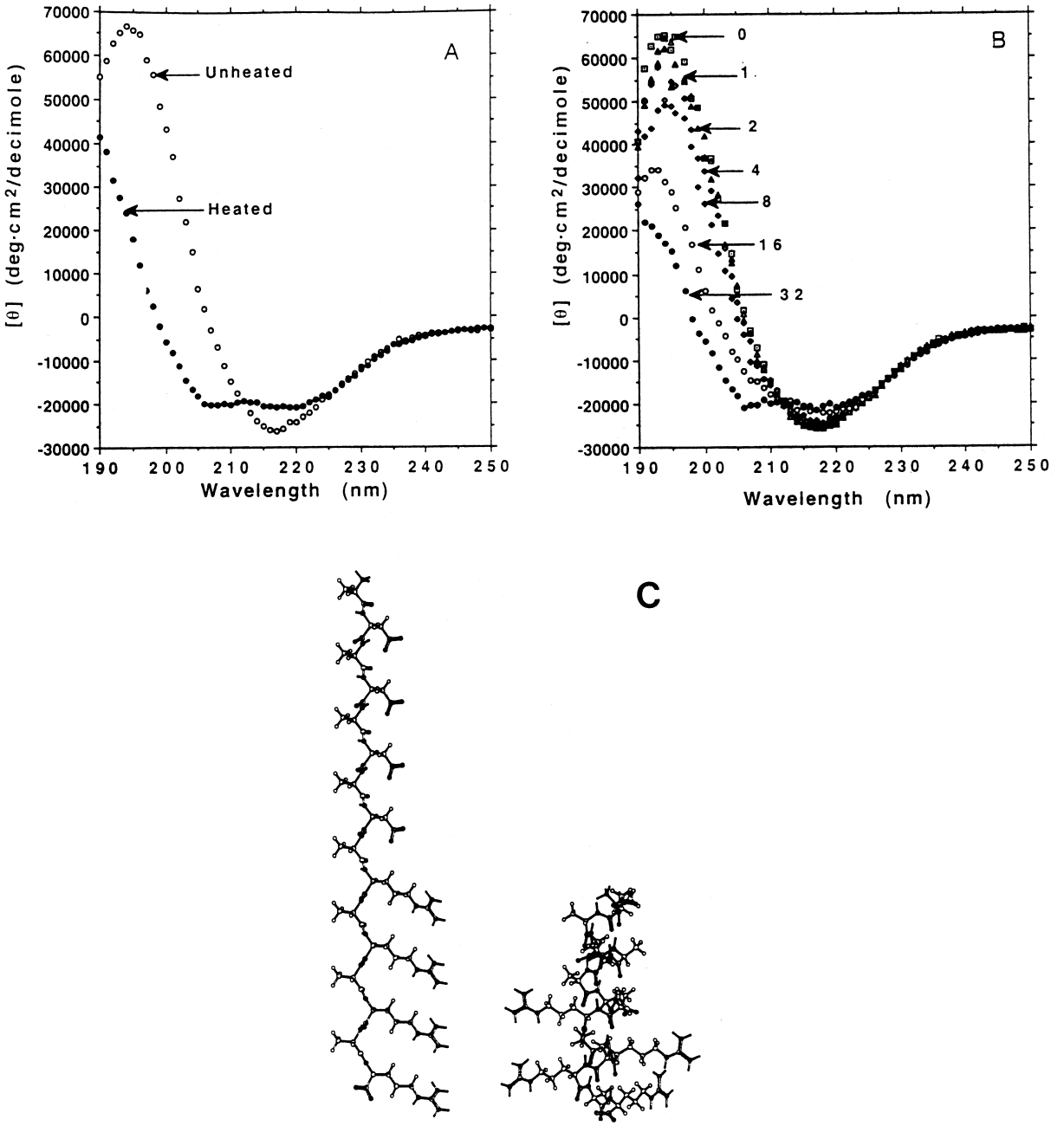

Fig. 4. Temperature induced secondary structural transition in DAR16-IV. (A) CD spectra were measured at 20°C with an identical sample heated at 90°C for 10 min. The heated sample has an apparent alpha-helical CD spectrum as indicated. (B) Seven identical samples of DAR16-IV were heated at 75°C for different time periods in minutes as indicated and returned to 23°C. Small differences are seen in the spectra between unheated and those heated for 1, 2, and 4 min. The transition began 8 min after heating and was completed after 32 min heating. No further changes were observed for additional time of heating. (C) Molecular models of DAR16-IV in a beta-strand form and an alpha-helical form. The length of the beta-strand is about 5 nm and the length of the alpha-helix is about 2.5 nm. Therefore, beta-form can be viewed as ON and the alpha-form as OFF.

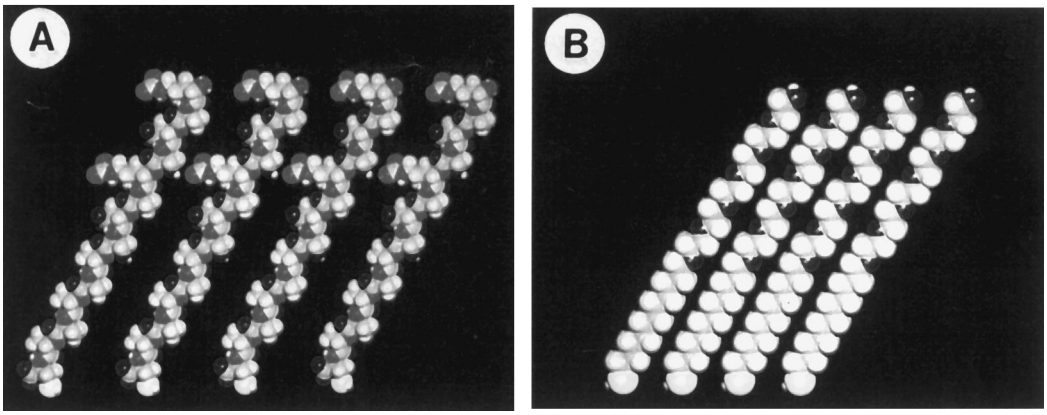

Fig. 5. Self-assembling peptides for biological surface engineering. Molecular models of the oligopeptide RADSC-14 with the sequence RADSRADSAAAAAC (A) and of ethylene glycol thiolate (EG6SH) (B). The N-terminal segment $(RADS)_2$ is the ligand for cell attachment, the five-alanine segment, AAAAA, is a linker to the anchoring cysteine. The cysteine anchor is covalently bound to the gold atoms on the surface. Molecular models are shown the surface where both molecules form self-assembled monolayers with different height. The extended lengths of RADSC-14 and EG6SH are approximately 5 and 4 nm, respectively.

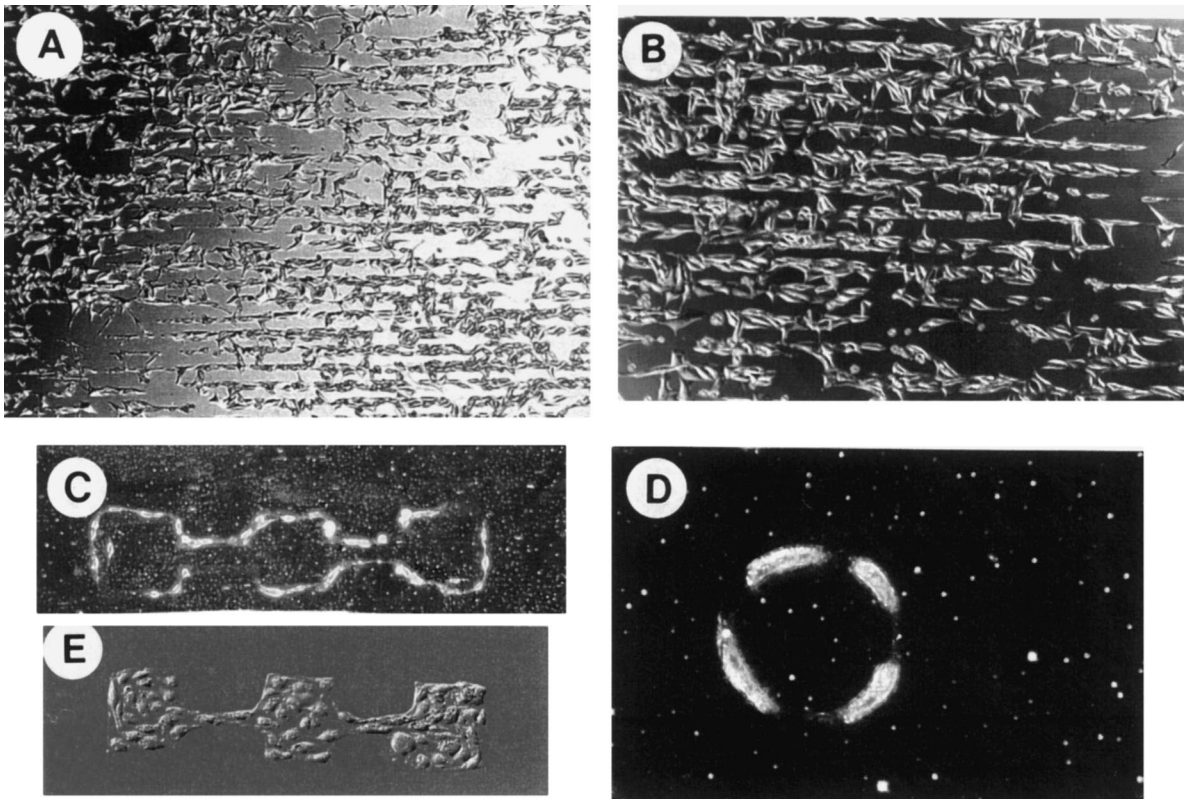

Fig. 6. Mouse fibroblast NIH 3T3 cell arrays. (A) The images of cells were taken with a Normarski microscope at 50X, (B) at 100X. (C) Bovine aortic endothelial cells formed the patterns of squares connected with linear cell tracks in line form and (E) in patch form. (D) Four individually separated cells formed a circle.

tured on a chip for rapid and sensitive detection. Furthermore, μCP using self-assembling peptides is simply repetitious and, therefore, a robotic system can be potentially developed for printing specifically designed pattern on chips or other surfaces. Such a system may be generally useful for rapid and high throughput drug screens that influence cell behavior.

This study is at the interface of several disciplines including biological materials science, surface self-assembly engineering, and biology. It is significant for four reasons: (1) it is an example of the applications of molecular engineering of biological substrates to studying cell-material interaction. These types of studies will clarify how cells interact with surface substrates, and the molecular interactions of cells with their surface environments. (2) Because we can design specific surface patterns, we can control specific cell–cell interactions. This surface engineering using combination of self-assembling oligopeptides and μCP will provide the opportunity to design many patterned surfaces. (3) Using this technique, we can study cell–cell communication in a well-controlled manner. This kind of systematic study will likely open new avenues to design higher order of architectures. (4) Microcontact printing using self-assembling peptides is a process that is readily amenable to automation.

5. Conclusion remarks

Development of new techniques often broadens the questions we can address and may thus deepen our understanding of seemingly intractable biological phenomena. Using nano-self-assembling peptide system, we can dissect complex problems into smaller units to study them systematically. We believe that application of these simple and versatile systems using these self-assembling oligopeptides will make it possible to study some complex and previously intractable biology phenomena, such as the study of cell–material interactions, detailed mechanism of cell migration, cell mechanical compliance, cell–cell communication, and community cell behavior. Biopolymer engineering through molecular design using various self-assembly of biological building blocks is an enabling technology that will likely play an increasingly important role in the future technology and will change our lives in the coming century.

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