

# Fabrication of molecular materials using peptide construction motifs

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**Biotechnology has generally been associated with gene cloning and expression, genomics, high throughput drug discovery, biomedical advancement and agricultural development. That is about to change. Biotechnology will expand to encompass discovery and fabrication of biological and molecular materials with diverse structures, functionalities and utilities. The advent of nanobiotechnology and nanotechnology have accelerated this trend. Analogous to the construction of an intricate architectural structure, diverse and numerous structural motifs are used to assemble a sophisticated complex. Nature has selected, produced and evolved numerous molecular architectural motifs over billions of years for particular functions. These molecular motifs can now be used to build materials from the bottom up. Biotechnology will continue to harness nature's enormous power to benefit other disciplines and society as a whole.**

Biotechnology as an industry is less than 30 year old. It emerged from a laboratory curiosity of elite scientific pursuit to a formidable technologically intense industry that has attracted hundreds of billions of dollars of investment in a continuous trend. Biotechnology has not only produced numerous products but also benefited society in fundamental ways that we could not have imagined as little as a generation ago. There is no doubt that biotechnology is still expanding at an accelerating rate and the best is yet to come. But what direction will the expansion take?

One of the trends is production of new molecular-scale materials that have become increasingly important for the next generation of biotechnology products. Essentially this is the design, synthesis and fabrication of nano-devices at the molecular scale from bottom up. Nature has already produced numerous and diverse building motifs through billions of years of molecular selection and evolution [1,2]. Basic engineering principles for micro- and nano-fabrication can now be learned through understanding molecular self-assembly and programmed assembly phenomena. Self- and programmed-assembly phenomena are ubiquitous in nature. The key elements in molecular self-assembly are chemical complementarity and structural compatibility through noncovalent weak interactions.

Several self-assembling peptide systems have been developed, ranging from models for studying protein folding and protein conformational diseases, to molecular materials for producing peptide nanofibers, peptide scaffolds, peptide surfactants and peptide ink [3,4]. These self-assembling peptide systems are simple, versatile, affordable and easy to produce at a large scale to drive the development of this new industry. These self-assembly systems represent a significant advance in molecular engineering for diverse technological innovations. Here, we focus on our work from the past decade. But those who are interested in trends over a longer period of time are referred to earlier reviews [4–6].

Molecular self-assembly is ubiquitous in nature and has recently emerged as a new approach in chemical synthesis, nanotechnology, polymer science, materials and engineering. Molecular self-assembly systems lie at the interface between molecular biology, protein science, biochemistry, polymer science, materials science and engineering [7,8]. Many self-assembling systems have been developed. Molecular self-assembly systems represent a significant advance in the molecular engineering of simple molecular building blocks useful for a wide range of applications (Figure 1). This field is growing at an accelerating pace riding on the tide of biotechnology.

## Peptides as construction motifs

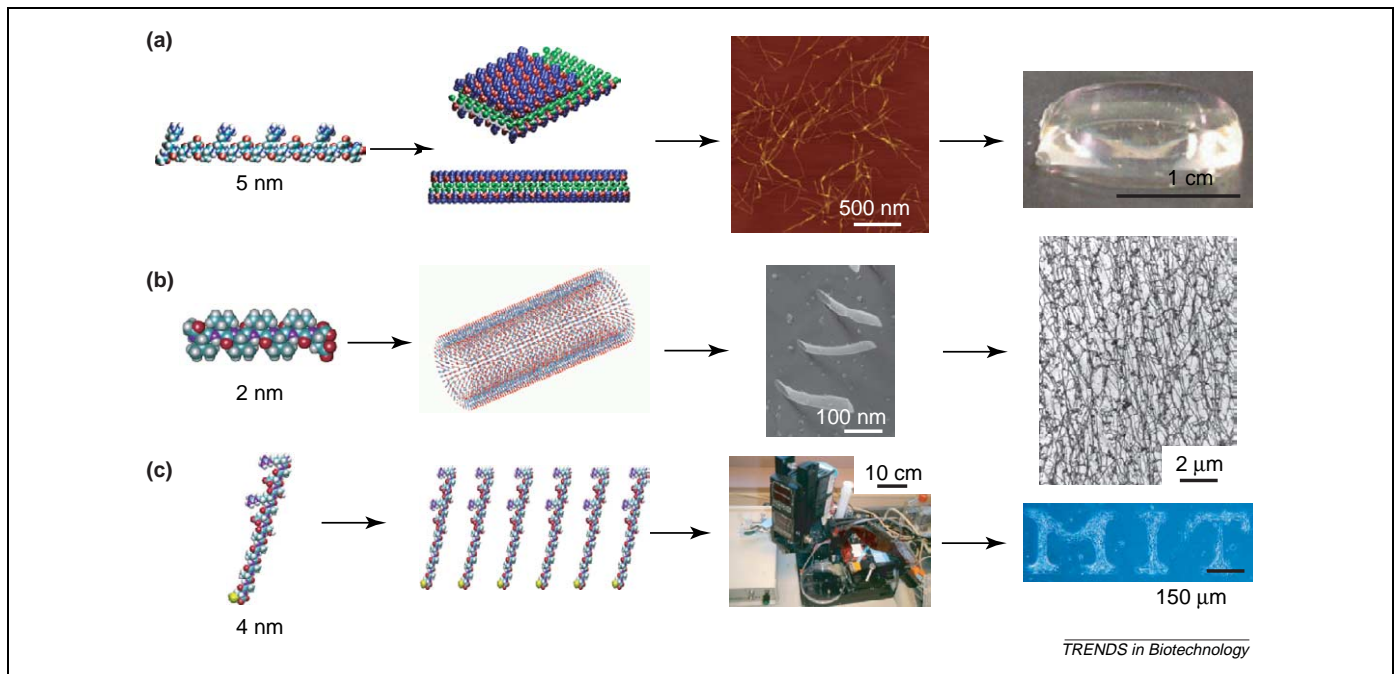
Similar to the construction of a house, doors, windows, and many other parts of the house can be prefabricated and programme-assembled according to architectural plans. If we shrink the construction units many orders of magnitude to the nanoscale, we can apply similar principles for constructing molecular materials and devices, through molecular self-assembly and programmed molecular assembly. Due to space constraints, only three self-assembling construction units are summarized here. They are: 'peptide Lego<sup>®</sup>' that forms well-ordered nanofiber scaffolds for 3D cell culture and for regenerative medicine; 'peptide surfactants or detergents' for drug, protein and gene deliveries as well as for solubilizing and stabilizing membrane proteins; and 'peptide ink' for surface biological engineering. These designed construction peptide motifs are structurally simple and versatile for a wide spectrum of applications.

## Peptide Lego<sup>®</sup>

Molecular-designed 'peptide Lego<sup>®</sup>', at the nanometer scale, resembles the Lego bricks that have both pegs and

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Available online 30 July 2004



**Figure 1.** Fabrication of various peptide materials. **(a)** Peptide Lego<sup>®</sup>, also called ionic self-complementary peptide has 16 amino acids, ~5 nm in size, with an alternating polar and nonpolar pattern. They form stable  $\beta$ -strand and  $\beta$ -sheet structures, thus the side chains partition into two sides, one polar and the other nonpolar. They undergo self-assembly to form nanofibers with the nonpolar residues inside (green) and positive (blue) and negative (red) charged residues forming complementary ionic interactions, like a checkerboard. These nanofibers form interwoven matrices that produce a scaffold hydrogel with very high water content, >99.5% water (images courtesy of Hidenori Yokoi). **(b)** Peptide surfactant/detergent, ~2 nm in size, have a distinct head group, either positively charged or negatively charged, and a hydrophobic tail consisting of six hydrophobic amino acids. They can self-assemble into nanotubes and nanovesicles with a diameter of ~30–50 nm (image courtesy Steve Santoso and Sylvain Vauthey). These nanotubes go on to form an inter-connected network, which has been observed in other nanotubes. **(c)** Peptide ink. This type of peptide has three distinct segments: a functional segment where it interacts with other proteins and cells; a linker segment that is either flexible or stiff and sets the distance from the surface, and an anchor for covalent attachment to the surface. 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. Neural cells from rat hippocampal tissue form defined patterns. (Images courtesy Sawyer Fuller and Neville Sanjana).

holes in a precisely determined organization that can be programmed to assemble in well-formed structures. This class of 'peptide Lego<sup>®</sup>' can spontaneously assemble into well-formed nanostructures at the molecular level [9]. The first member of the peptide lego was serendipitously discovered from a segment in a left-handed Z-DNA binding protein in yeast and named Zuotin (Zuo means left in Chinese, tin means protein in biology) [10].

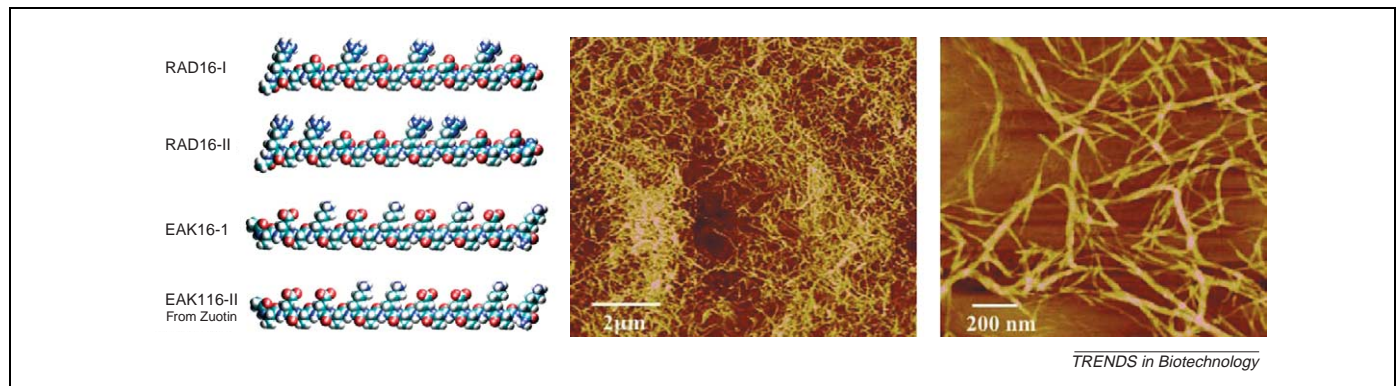
These peptides form  $\beta$ -sheet structures in aqueous solution thus they form two distinct surfaces, one hydrophilic, the other hydrophobic, like the pegs and holes in Lego<sup>®</sup> bricks. The hydrophobic sides shield themselves from water allowing them to self-assemble in water, as seen in spontaneous protein folding. The unique structural feature of these 'peptide Lego<sup>®</sup>' is that they form complementary ionic bonds with regular repeats on the hydrophilic surface (Figure 2). 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 positive and negative charged amino acid residues, with intervals of 1, 2, 3, 4 amino acids, and so on. For example, charge arrangements are for modulus I,  $- + - + - + - +$ ; modulus II,  $- - + + - - + +$ ; modulus III,  $- - - + + +$ ; and modulus IV,  $- - - - + + + +$ . The charge orientation can also be designed in the reverse orientation that can yield entirely different molecules. These well-defined sequences allow the molecules to undergo ordered self-assembly, resembling some situations found in well-studied polymer assemblies [3,4,11].

The peptide Lego<sup>®</sup> molecules can undergo self-assembly in aqueous solutions to form well-ordered nanofibers that further associate to form nanofiber scaffolds [9,12–14]. One of them, RADA16-I, is called PuraMatrix, because of its purity as a designed biological scaffold in contrast to other biologically derived scaffolds from animal collagen, and Matrigel containing unspecified components in addition to known materials.

Because these nanofiber scaffolds have 5–200 nm pores and have an extremely high water content (>99.5% or  $1\text{--}5\text{ mg ml}^{-1}$ ) (Figure 2), they are useful in the preparation of 3D cell-culture media. The scaffolds closely mimic the porosity and gross structure of extracellular matrices, allowing cells to reside and migrate in a 3D environment, and molecules, such as growth factors and nutrients, to diffuse in and out very slowly. These peptide scaffolds have been used for 3D cell culture, controlled cell differentiation, tissue engineering and regenerative medicine applications [12,13,15,16].

### Peptide surfactants or detergents

We designed a class of peptide surfactants having hydrophobic tails and hydrophilic heads, taking advantage of their self-assembly properties in water [17–19]. Several peptide surfactants have been designed using nature's lipid as a guide. These peptides have a hydrophobic tail with various degrees of hydrophobicity and a hydrophilic head, either negatively charged aspartic and glutamic acids or positively charged lysine or histidine (Figure 3). These peptide monomers contain 7–8 amino



**Figure 2.** Molecular models of several self-assembling peptides, RAD16-I, RAD16-II, EAK16-I and EAK16-II. Each molecule is  $\sim 5$  nm in length with 8 alanines on one side and 4 negative and 4 positive charged amino acids in an alternating arrangement on the other side. AFM image of RADA16-I nanofiber scaffold (PuraMatrix). Note the nanofiber scale fiber with pores ranging from 5–200 nm, the correct pore size for biomolecular diffusion. This is in contrast to the microfibers of traditional polymer scaffolds, in which the fiber diameter is  $\sim 10$ –50 micron and the pores range from 10–200 microns.

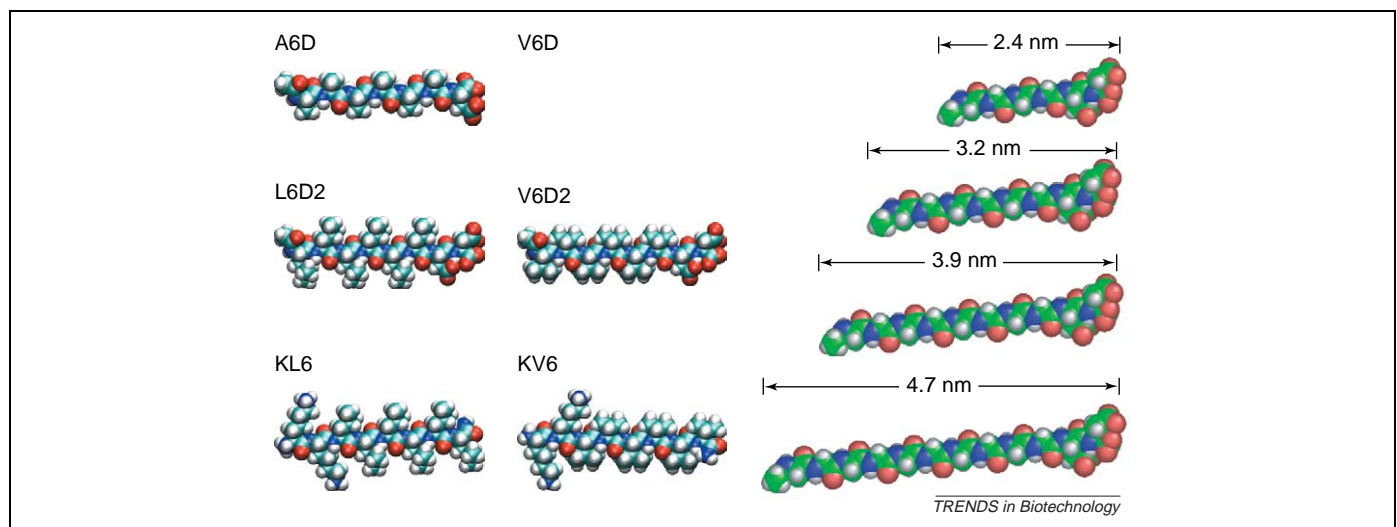
acid residues and have a hydrophilic head composed of aspartic acid and a tail of hydrophobic amino acids, such as alanine, valine or leucine. The length of each peptide is  $\sim 2$  nm, similar to that of biological phospholipids [17,19]. The length can also be varied by adding more amino acids, one at a time to a desired length as shown in Figure 3 [18].

Although individually these peptide surfactants have completely different composition and sequences, these peptides share a common feature: the hydrophilic heads have 1–2 charged amino acids and the hydrophobic tails have four or more consecutive hydrophobic amino acids. For example,  $A_6D$  (AAAAAAD),  $V_6D$  (VVVVVD) peptide has six hydrophobic alanine or valine residues from the N-terminus followed by a negatively charged aspartic acid residue, thus having two negative charges, one from the side chain and the other from the C-terminus. Likewise,  $G_8DD$  (GGGGGGGDD), has eight glycines following by two aspartic acids with three negative charges. By contrast,  $K_2V_6$  (KKVVVVV) has two positively charged lysines as the hydrophilic head, followed by six valines as the hydrophobic tail [17–19].

These peptides undergo self-assembly in water to form nanotubes and nanovesicles having an average diameter of 30–50 nm [17–19] (Yang and Zhang, unpublished). The tails consisting of alanines and valines produce more homogeneous and stable structures than those of glycines, isoleucine and leucine. This property might be due to their hydrophobic and hydrophilic ratios. These monomer surfactant peptides were used for molecular modeling. The negatively charged aspartic acid is modeled as red and positively charged lysine is blue with the green as the hydrophobic tails. They form tubular and vesicle structures.

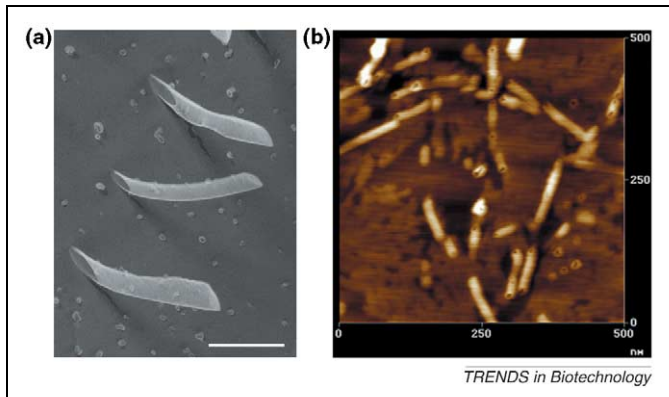
Quick-freeze/deep-etch sample preparation where the sample was flash-frozen at  $-190^\circ\text{C}$  instantly produced a 3D structure with minimal structural disturbance. It revealed a network of open-ended nanotubes using transmission electron microscopy (Figure 4) [17–19]. There seemed to be dynamic molecular behavior overtime. Likewise,  $A_6K$  cationic peptide also exhibited similar nanotube structures with the opening ends clearly visible [17, Yang and Zhang, unpublished results].

It is interesting that these simple surfactant peptides can produce remarkable complex and dynamic structures.



**Figure 3.** Molecular models of peptide surfactants/detergents. Left panel.  $A_6D$ ,  $V_6D$ ,  $V_6D_2$  and  $L_6D_2$ .  $KKL_6$ ,  $KKV_6$ . D (Aspartic acid) bears negative charges and A (alanine), V (valine) and L (leucine) constitute the hydrophobic tails with increasing hydrophobicity. Each peptide is  $\sim 2$ –3 nm in length, similar to biological phospholipids. K (lysine). Right panel. Molecular structures of individual glycine tail-based surfactant peptides.  $G_4D_2$ ,  $G_6D_2$ ,  $G_8D_2$  and  $G_{10}D_2$ . The tail length of glycines varies depending on the number of glycine residues. The lengths of these molecules in the extended conformation range from 2.4 nm of  $G_4D_2$  to 4.7 nm of  $G_{10}D_2$ . Color code: carbon, green; hydrogen, white; oxygen, red; and nitrogen, blue.





**Figure 4.** Quick-freeze/deep-etch TEM image of  $V_6D$  dissolved in water (4.3 mM at pH 7) at high-resolution and AFM image of  $A_6K$ . (a) The images show the dimensions of nanotube ends (30–50 nm in diameter, with openings). Note opening ends of the peptide nanotube can be cut vertically. The strong contrast shadow of the platinum coat also suggests the hollow tubular structure. There are openings at one end with the other ends possibly buried. The diameter of the  $V_6D$  nanobutes is  $\sim$ 30–50 nm. Scale bar 100 nm. (b) The nanotubes of  $A_6K$  peptide surfactant/detergent. The openings are clearly visible in the AFM image. Note that  $V_6D$  and other anionic peptide surfactants cannot be imaged on negatively charged mica surfaces with AFM because they do not adhere well to mica.

This is another example of building materials from the bottom up.

How could these simple surfactant peptides form such well-ordered nanotubes and nanovesicles (Figure 5). There are molecular and chemical similarities between lipids and these peptides because both have a hydrophilic head and a hydrophobic tail. However, the packing between lipids and peptides is likely to be quite different. In lipids, the hydrophobic tails pack tightly against each other to completely displace water, precluding the formation of hydrogen bonds. In addition to hydrophobic tail packing between the amino acid side chains, surfactant peptides also interact through intermolecular hydrogen bonds along the backbone.

These peptide surfactants or detergents have been found to be excellent materials for solubilizing, stabilizing (Zhao, Kiley, Nagai, Yeh and Zhang, unpublished) and crystallizing several diverse membrane proteins. These simply designed peptide detergents could open a new avenue to overcome one of the biggest challenges in biology – obtaining high resolution structures of membrane proteins. The study of membrane proteins will not

only enrich and deepen our knowledge of how cells communicate with their surroundings, thus how all living systems respond to their environments, but these membrane proteins can also be used to fabricate the most advanced molecular devices, from energy harnessing devices, extremely sensitive sensors to medical detection devices and surely applications that we cannot image at present.

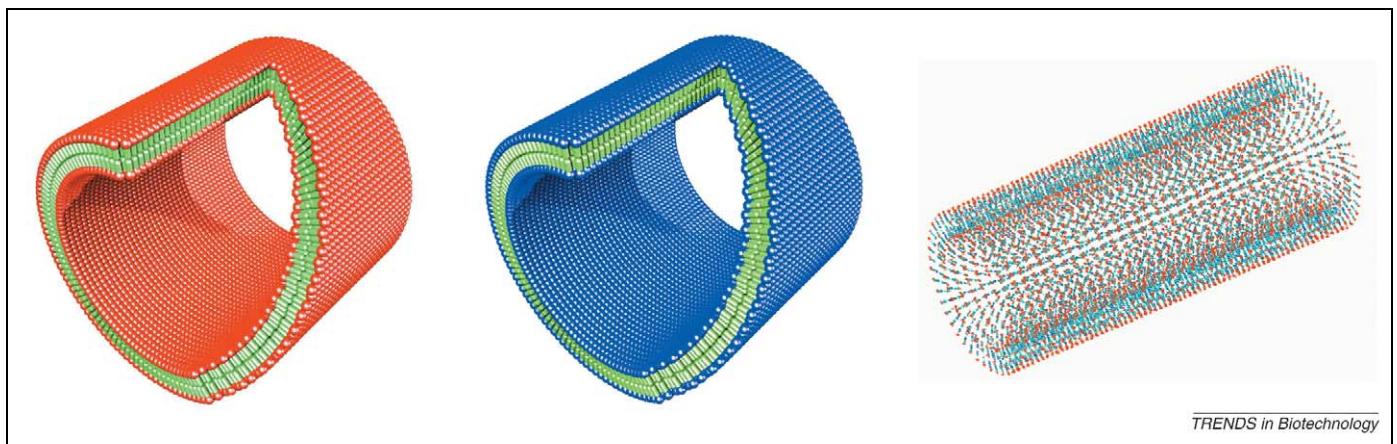
Furthermore, several cationic peptide surfactants have been tested for their ability to encapsulate DNA and deliver DNA into cells (von Maltzahn and Zhang, unpublished).

### Peptide ink

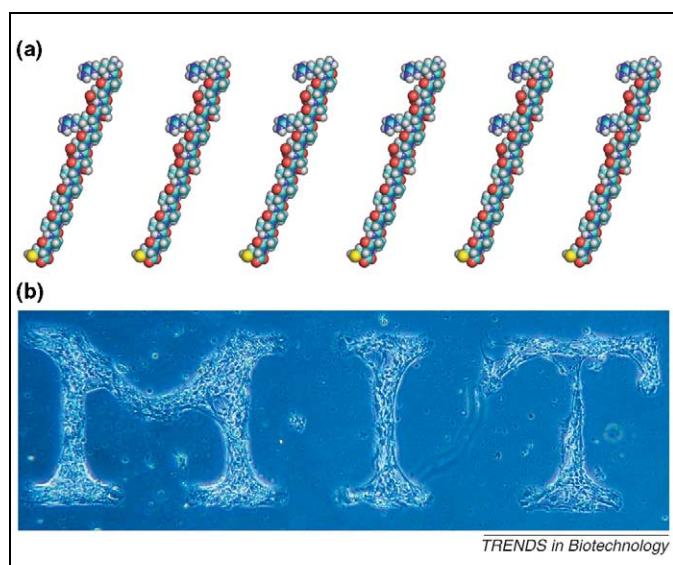
Whitesides and coworkers developed a microcontact printing technology that combines semi-conducting industry fabrication, chemistry, and polymer science to produce defined features on a surface down to the micrometer or nanometer scale [7,20–21]. Following microcontact printing, a surface can be functionalized with different molecules using a variety of methods, such as covalent coupling, surface adhesion and coordination chemistry. Surfaces have now been modified with a variety of chemical compounds. Furthermore peptides and proteins as inks have also been printed onto surfaces. This development has spurred new research into the control of molecular and cellular patterning, cell morphology, and cellular interactions, and fueled new technology development.

Molecular surface assembly can be targeted to alter the chemical and physical properties of a material's surface. Surface coatings instantly alter a material's texture, color and compatibility with and responsiveness to the environment.

Peptide or protein inks have been directly printed on surfaces to allow adhesion molecules to interact with cells and adhere to the surface (Figure 6). These peptides have three general regions along their lengths: (i) a ligand for specific cell recognition and attachment; (ii) a linker for physical separation from the surface; and (iii) an anchor for covalent attachment to the surface [22–23]. The ligands might be of the RGD (arginine–glycine–aspartic acid) motif that is known to promote cell adhesion, or other



**Figure 5.** Molecular models of peptide surfactant/detergent nanobutes. Color code: hydrophobic tail, green; negatively charged head ( $V_6D$ ), red; and positively charged head ( $V_6K$ , or  $KV_6$ ), blue.



**Figure 6.** Molecular structure of peptide ink. (a) This class of peptide ink has three general regions along their lengths: a ligand for specific cell recognition and attachment, a linker for physical separation from the surface, and an anchor for covalent attachment to the surface. Color code: carbon, green; hydrogen, white; oxygen, red; nitrogen, blue; thiol group, yellow. (b) Cells adhere to printed patterns. The protein was printed onto a uniform PEG inhibitory background. Cells adhered to patterns after 8–10 days in culture spelling MIT. (Images courtesy Sawyer Fuller and Neville Sanjana).

sequences for specific molecular recognition, or specific cell interactions. The linker is usually a string of hydrophobic amino acids such as alanine or valine. The anchor can be a cysteine residue for gold surfaces, aspartic acid linking on amine surfaces, or lysine linking on carboxylic surfaces.

This approach could facilitate research into cell–cell communication. Recently, we have moved one step further: using proteins or peptides as ink we have directly microprinted specific features onto the non-adhesive surface of polyethylene glycol to write any arbitrary patterns rapidly without preparing the mask or stamps (Figure 6). The process is similar to using an ink pen for writing – here, the printing device is the pen and the biological substances are the inks.

This simple and rapid printing technology allowed us to design arbitrary patterns to address questions in neurobiology that would not have been possible before.

Because understanding of correct complex neuronal connections is absolutely central to comprehension of our own consciousness, human beings are always interested in finding ways to further investigate this. However, the neuronal connections are exceedingly complex, we must dissect the complex neuronal connections into smaller and more-manageable units to study them in a well-controlled manner through systematic biomedical engineering approaches. Therefore, nerve fiber guidance and connections can now be studied on special engineered pattern surfaces that are printed with protein and peptide materials. The surface substrate can either be from purified native proteins or from self-assembling peptide-containing cell adhesion motifs, prepared through systematic molecular engineering of amino acids. Previous studies have shown that nerve fibers attach and outgrow extensively on the self-assembling peptide matrices [13].

We are interested in studying the nerve fiber navigation on designed pattern surfaces in detail. Studies of nerve fiber navigation and nerve cell connections will undoubtedly enhance our general understanding of the fundamental aspects of neuronal activities in the human brain and brain–body connections. It will probably also have applications in screening neuropeptides and drugs that stimulate or inhibit nerve fiber navigation and nerve cell connections.

### Peptide surfactants stabilize membrane proteins

Although membrane proteins make up approximately one-third of total cellular proteins and carry out some of the most important functions in cells, only several dozen membrane protein structures have been elucidated. This is in striking contrast to ~25 000 non-membrane protein structures that have been solved [24,25]. The main reason for this delay is the difficulty in purifying and crystallizing membrane proteins because removal of lipids from membrane proteins affects protein solubility and conformational stability. Despite the wide variety of detergents and lipids as surfactants that have been used to allow solubilization, stabilization, purification and crystallization of proteins over several decades, the way surfactants interact with membrane proteins to influence structure and function and therefore criteria for choosing good surfactants remain largely unknown. This is partly due to the complexity of membrane protein–detergent–lipid interactions and lack of a ‘magic material’ surfactant. Hence the need to develop new membrane compatible material is acute.

### Other peptide construction motifs as material building blocks

Aggeli and colleagues reported formation of nanofibers self-assembled from several peptides that leads to ~10 nm fibrils with differing degrees of left-handed helical twist [26].

Several chemical engineers and chemists have also fabricated artificial amphiphilic proteins using repetitive peptide motifs as the construction units [27–31]. They used diverse designed peptide motifs to arrange them in different ways and to fabricate a variety of novel materials. The resulting designed multiple peptide motifs as artificial proteins have considerable mechanical strength and fast-recovering scaffolds that are responsive to changes in pH and can resist temperatures of up to 90 °C [27–31].

Building blocks beyond natural peptide construction motifs can be used to create peptide materials. Stupp and colleagues [32,33] have designed a ‘peptide-amphiphile’ derivitized with non-polar hydrocarbon tails, demonstrating that a composite of hydrophobic polymer tails and hydrophilic peptide heads can be used to form nanocylinders. These molecules have a long alkyl chain at the N-terminus of a peptide that acts as the hydrophobic tail. By incorporating a phosphorylated serine within the peptide end of the molecule calcium or other ions can be attracted, organized, and regulated to facilitate the mineralization of hydroxyapatite [32,33]. The C-terminus of the peptide can be further functionalized with the RGD



motif, thereby promoting cell attachment onto the nanofibers.

Reches and Gazit have demonstrated using the shortest peptide, a Phe–Phe dipeptide, can form stable nanotubes [34]. By diffusing silver ions into the well-formed tubes, then removing the peptide either enzymatically, chemically, or through heat burning, the silver wire was revealed [34].

Amyloid protein nanofibers have also been used as scaffold to align gold nanocrystals. Scheibel, Lindquist and colleagues report that a bioengineered prion-determining (NM) domain of yeast prion protein Sup35 provided a scaffold for fabricating nanowires. They also tested the conducting capability of the resulting wires [35].

Belcher and colleagues take a very different approach, not only to discover but also to fabricate electronic and magnetic materials, departing sharply from the traditional materials process technology [36–38]. Their strategy is to genetically engineer self-assembling bacteriophage so that they can be used to select conducting, semi-conducting and magnetic materials. They have also evolved the phages (and other microbial organisms) for additional material fabrications. This strategy might lead to the discovery of new electronic and magnetic materials [35–37].

### Future trends in biotechnology

One of the trends in biotechnology is the development of new biological materials and biotechnologies that in turn often broaden the questions we can address therefore deepening our understanding of seemingly intractable biological phenomena. Self-assembling peptide systems will create a new class of materials at the molecular scale and will have a high impact in many fields.

One of the benefits of studying self-assembling peptide systems unexpectedly emerged recently. We found that the simple peptide surfactant/detergents (Figure 4) can be excellent materials for solubilizing and stabilizing membrane proteins that have been extremely difficult to work with and lag far behind in solving their high-resolution structures. In one case, a membrane protein was crystallized in a very short time. This surprising finding encouraged us to turn our attention to work on membrane proteins that we would not have considered before.

We believe that application of these simple and versatile molecular self-assembly systems will provide us with new opportunities for studying complex and previously intractable biological phenomena. Molecular engineering through molecular design of self-assembling peptides is an enabling technology that will likely play an increasingly important role in the future of biotechnology and will change our lives in the coming decades.

We have encountered many surprises since we started our serendipitous journey of working on various self-assembling peptide systems: from developing a class of pure peptide nanofiber scaffolds for 3D tissue culture and for tissue engineering [12,13,15,16,38], studying of the model system of protein conformational diseases [39–42], and designing peptide or protein inks for surface printing [22,23] to finding peptide surfactant/detergents that solubilize and stabilize membrane proteins. As Sir John

Maddox best put it ‘And who now, dares say that the days of surprises are over?’ [43].

### Acknowledgements

We thank members of the laboratory, past and present, for making discoveries and conducting exciting research. We gratefully acknowledge the supports by grants from ARO, ONR, DARPA (BioComputing), DARPA/Naval Research Laboratories, DARPA/AFO, MURI/AFO, NIH, NSF-MIT BPEC and NSF CCR-0122419 to MIT Media Laboratory's Center for Bits and Atoms, the Whitaker Foundation, DuPont-MIT Alliance, and Menicon, Ltd, Japan. We also acknowledge the Intel Corporation educational donation of a computing cluster to the Center for Biomedical Engineering at MIT.

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