RATIONAL DESIGN OF PEPTIDE-BASED SUPRAMOLECULAR POLYMERS AS FUNCTIONAL BIOMATERIALS

by

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Abstract

Ordered supramolecular polymers are one-dimensional (1D) nanostructures formed by spontaneous association of molecular building units through non-covalent interactions. The construction of supramolecular polymers, often classified as a bottom-up approach, involves the self-organization of smaller building units into hierarchically complex structures. This approach has led to the development of synthetic materials that serve as extraordinary candidates for use in energy and medicine. To rationally design functional materials with desired properties, the critical feature ultimately lies in the design parameters of the constituents that determine the intermolecular interactions and affect the assembling behaviors or functions. In-depth understanding of such systems is therefore crucial to create self-assembled materials that overcome current and future challenges in their applications.

Peptide-based molecules offer an excellent synthetic platform to fabricate such supramolecular polymers through self-assembly in aqueous environments. Although self-assembly of amphiphilic peptides containing a β-sheet rich segment into 1D structures has been well-documented, little is known as to how the molecular architecture of the building blocks affects the self-organization into different types of 1D assemblies (such as nanofibers, nanoribbons, or nanotubes). For instance, branched chemical structures, though often considered as a flexible design for developing multifunctional block copolymers, are rarely introduced in peptidic systems. This thesis is aimed to develop peptide-based functional materials with potential applications in
drug delivery or as cell scaffolding materials, by elucidating the structure-property relationship of the building blocks with the resulting macromolecular materials. Primarily focusing on the fabrication of 1D structures, we demonstrate the possibility of using molecular design to control three critical features of ordered supramolecular polymers as biomaterials: 1) the morphology of the assemblies, 2) the enzymatic degradability, and 3) the rheological properties.

We first investigated the requirements for designing nanotubes/nanofibers self-assembled by drug-peptide conjugates. Conjugation of an anticancer drug, camptothecin (CPT), into a peptidic segment enables the molecule to assemble into 1D structures given the strong directional interactions (hydrogen bonds or π-π stacking) among the building units. It was found that through the use of catanionic mixing or metal-coordination, the induced change of packing geometry of building units could determine whether these drug-peptide conjugates eventually assemble into nanotubes or nanofibers during 1D growth. In these cases, the change from a more conical-like to a less conical-like geometry could be tuned by the formation of ion-paired amphiphiles in catanionic mixing, or by coordination between designed hydroxamate groups and Fe(III) ions. This change in molecular packing could dictate the self-assembly from forming nanofibers into nanotubes. This discovery offers insight into rational design of proper peptidic molecules with predictable assembling morphologies.

Secondly, a platform of branched peptides was introduced, and we exploit this particular molecular design to fabricate filamentous networks for two purposes. The first example is the combined use of this molecular design and a crosslinking strategy to
develop modeling materials for mimicking the extracellular matrix (ECM), incorporated with matrix metalloproteinase (MMP) specific degradability. This strategy was initiated through the design of an amphiphilic peptide that could undergo a rapid morphological transition in response to pH variations—where the assembled filaments existed in pH 4.5 but quickly dissociated in pH 7.5. And then MMP specific peptide substrates were introduced as crosslinkers to covalently fix the filaments in the self-assembled state. The crosslinked filaments were stable at pH 7.5, but gradually broke down into much shorter filaments upon cleavage of the peptidic crosslinkers by MMP. This platform is believed to be useful for the creation of supramolecular filaments responsive to enzymatic degradation. The next example continued the use of the branched construct for designing isomeric molecules to fine-tune the local viscoelastic properties of supramolecular polymers while maintaining similar surface chemistry and mesh size. The stiffness of 3D matrices, probed by particle-tracking microrheology, could be correlated with the degree of molecular packing order within the hydrophobic cores of the 1D assemblies.

The findings featured in this thesis provide in-depth understanding of the key role of the molecular design parameters in defining their functions, assembling behaviors, and potential applications in chemical, biomolecular and biomedical engineering.

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1 Introduction

The spontaneous association of small molecules into discrete nanostructures has drawn many researchers’ attention for decades and developed into a rapidly progressing field.\textsuperscript{1-3} This strategy, often referred to bottom-up approach, literally involves piecing small building units together and then generating complex structures.\textsuperscript{1-5} The self-assembly process of small molecules into supramolecular structures, driven by interactions among the building units, is strongly linked with the molecular structures of individual units. From the aspect of applications, this bottom-up approach serves as an effective means to construct nanomaterials at the order of a few nanometers to hundreds of nanometers, providing an efficient methods to produce nanomaterials at large scale, which also serves as a powerful tool to control the surface chemistry, morphology, and functionality of nanomaterials by properly designing the building units.

The spontaneous processes of the all self-assembling occurrences are explained by the non-covalent intermolecular interactions among the building blocks. These noncovalent interactions can be van der Waals interaction, electrostatic interaction, hydrophobic interaction, hydrogen bond, or coordination bond.\textsuperscript{3,6} It becomes a specific branch in chemistry fields termed \textit{supramolecular chemistry} that aims to understand the role of intermolecular interactions affecting the self-assembling processes. The importance of supramolecular chemistry was initially brought to public attention when Jean-Marie Lehn, Donald J. Cram, and Charles J. Pedersen were awarded with the Nobel Prize for Chemistry in 1987. This field has grown rapidly since then, drawing much
attention from many researchers. Knowledge in supramolecular chemistry not only shed light on designing the next generation of nanomaterials; but also helped to explain the innate properties of various phenomena in Nature and provide insight into the properties of many biomolecules and biomacromolecules. Following are a few examples where the self-assembly process is influential in Nature.

*Lipid bilayers*, for instance, are one of the most classic examples of self-assembled structures in Nature, whose major building blocks are the phospholipids (Figure 1-1). They comprise the shell of various viruses, the cell membrane of most living organisms, and the membranes of subcellular organelles (e.g., nucleus, mitochondria, or endoplasmic reticulum) in cells. The innate nature that leads to the self-assembly of phospholipids, in fact, is programmed in their chemical structures. Most of the phospholipids possess a hydrophilic headgroup and fatty acid tails as the hydrophobic tails in their chemical structures. This particular type of chemical structure represents a classic molecular construct that bears the *amphiphilicity* required for self-assembly. The term amphiphilicity here means part of the molecular segment is hydrophobic while another part is relatively hydrophilic (attracted to water). Such amphiphilic molecules are called amphiphiles. In this instance, the *hydrophobic interaction* is the main driving force that accounts for the association of phospholipids. The hydrophobic interactions originate from the fact that non-polar species have higher affinity toward non-polar substance. Therefore, the fatty acid tails of phospholipids possess a spontaneous tendency to shield themselves from the aqueous media and pack within hydrophobic environments. Being more attracted toward the polar aqueous environments, the hydrophilic headgroups
shield the hydrophobic domains from water, and the internal fatty acid tails behaves similarly to liquid crystalline phases. The resulting supramolecular structure in aqueous media, in this example, is a bilayer structure where the surface displays the hydrophilic headgroups.

Figure 1-1. Self-assembly of phospholipids, the major constituents of lipid bilayer and cellular membranes. a-b) Chemical structure of a phospholipid phosphatidylcholine (a) and a lipid bilayer formed by phospholipids in aqueous media where the hydrophobic tails are embedded within the shelter of hydrophilic headgroups (b). Adapted from ref (7). c) The mosaic model of cellular membranes whose major constituents are phospholipids. Adapted from ref (8).

Another example of self-assembly is the aggregation process of proteins. The self-assembling mechanism of forming amyloid fibrils has drawn much attention in order to elucidate the pathological process of misfolding proteins. Amyloid fibrils are highly ordered, insoluble, supramolecular structures that result from the spontaneous aggregation of inappropriately folded proteins that interact with one another.8-11 They have been shown to be associated with various neurogenic diseases, such as Parkinson’s
Results of spectroscopic analysis show that the secondary structures of proteins play a key role in the aggregation process; it was discovered that the formation of β-sheets are critical for the formation of amyloid fibrils (Figure 1-2). Electron microscopy further shows that these fibrils usually comprise several protofilaments, which eventually intertwine into long ribbons with ~30 nm widths. In general, the aggregation occurrences of such amyloid fibrils are strongly associated with the hydrophobicity and charges encoded in the amino acid sequences, which will be discussed in later content.

**Figure 1-2. Self-assembly of amyloid fibrils.** a) Modeled structure of monomer Aβ_{1-40} in β-strand conformation. Aβ_{1-40} is known as the key sequence that lead to the formation of amyloid fibrils in Alzheimer’s disease. b) Schematic representation of the formation of β-sheets in protein aggregation into fibril. c) Representative micrographs of amyloid fibrils in TEM microscopy. Images are adapted from ref (8).

In both given examples illustrated above, it is the non-covalent interactions that strongly determine the self-assembling behaviors of the building blocks. Inspired by the self-assembly process in naturally occurring systems, many researchers engineered nanomaterials based on this basic principal. The general rationale of designing self-assembling molecules basically involves manipulating the intermolecular forces of the
building units that encoded in the chemical structures. Overall amphiphilicity is one of the common requirements of designing molecules that spontaneously undergo self-assembly processes into discrete nanostructures. There are a plethora of examples employing synthetic methods to construct self-assembled structures by amphiphiles, including surfactants, block copolymers, or amphiphilic peptides or proteins. Despite much work in this field, the connections between the design parameters of building units and their resulting supramolecular properties are still not fully studied and explicitly understood.

In this thesis, the aim is to investigate the critical role of design parameters of amphiphilic peptide conjugates that ultimately affect the size, morphology, and function of the assemblies in aqueous media. In particular, we would like to explore the potential of these nanomaterials for applications as nanomedicine and cell scaffolds. It is hoped that the fundamental understanding of amphiphile design will add to the effective development of the next generation of functional biomaterials by using self-assembly.

1.1 Self-Assembly of Peptides and Peptide Conjugates into Supramolecular Polymers

Amphiphilic peptides/peptide conjugates are one class of effective building blocks for the construction of discrete nanostructures with controlled surface chemistry and functionality, ranging from micelles, vesicles, nanofibers, or nanotubes. Bearing much resemblance to the self-assembly processes of proteins, the resulting supramolecular morphology formed by these small biomolecules is notably dependent
on the intermolecular interactions that originate from the intrinsic properties of the peptides.

In this work, most of the covered contents will emphasize the use of peptide conjugates to construct self-assembled one-dimensional (1D) structures that are characterized by an extremely large aspect ratio. Thus, in this section, we would like to mention some examples of self-assembling peptides that form 1D supramolecular structures and how they were applied as functional materials. These 1D structures are often called “supramolecular polymers”, polymers whose monomers are merely joined with non-covalent interactions, contrasting with conventional polymers whose monomers are linked by covalent bonds.

In general, the self-assembly of amphiphilic molecules that leads to 1D supramolecular structures requires the presence of anisotropic interactions between the building blocks, such as hydrogen bonding or π-π interactions. This principal, in fact, not only accounts for the mechanism of the formation of amyloid fibrils (mentioned in Section 1), but also becomes an important criterion for designing monomeric molecules to construct supramolecular polymers. It was discovered that the formation of amyloid fibrils follows a process similar to nucleation-elongation mechanism in many neurogenic diseases. The self-aggregation process of peptides/proteins into long 1D structures is found strongly associated with the secondary structure, which is determined by the peptide sequence, as mentioned previously. Further studies show that peptides or proteins rich in β-sheet possess an extraordinary propensity to form intermolecular hydrogen bonds and often lead to the self-aggregation into 1D supramolecular filaments.
Evidence from X-ray scattering suggests the intermolecular hydrogen bonds run along the long axis of the fibrils. Due to this highly selective nature of forming 1D structures and also the ease of chemical modifications of peptides, the designing of synthetic peptides rich in \( \beta \)-sheet accordingly serves as powerful bottom-up strategy to construct supramolecular polymers with controlled chemical or biological properties, allowing the development of a great range of functional materials.

Stupp and coworkers have reported various types of peptide amphiphiles, molecules that possess a hydrocarbon segment and peptidic domain (Figure 1-3a). The designed peptide amphiphiles can easily assemble into nanofibers, similar to long cylindrical micelles, in aqueous media when \( \beta \)-sheet segments are incorporated. They greatly benefit from the ease with which the surface chemistry of the assembled structures can be controlled by conjugating bioactive epitopes to the hydrophilic domain of the molecules. This platform, where one can incorporate different types of bioactive epitopes on the surface of the nanofibers based on their desired function, has shown its potential in stem cell studies, regenerative medicine, and cancer therapy. Schneider and coworkers also proposed another family of self-assembling peptides that exhibit the capability to form 1D nanostructures in aqueous media (Figure 1-3b). Their building block possesses a hairpin-like design with alternating hydrophobic and hydrophilic amino acids. This design also possesses allows for the fabrication of functional scaffolds with interesting applications, possessing antimicrobial, biomineralization-directing, or anticancer properties.
1.2 Designing Nanomedicine for Self-Delivery Anticancer Therapeutics

Developing effective vehicles for the delivery of hydrophobic anticancer drugs to tumor sites has garnered major attention in cancer chemotherapies for decades.\textsuperscript{40-42} It is still an ongoing mission to seek advances in therapeutic efficacy, because most of the chemotherapeutic agents still bear relatively high toxicity and insufficiently discriminate between malignant cells and healthy cells. The idea of bringing nanotechnology into pharmaceutical sciences could develop into a promising field that may allow the current obstacles in cancer therapy to be overcome. There are many instances of using of drug-loaded nanocarriers to improve a drug’s pharmacokinetic properties and biodistribution profile, where the drug is carried and shielded by the delivery vehicle at the nanoscale, aiming to deliver the therapeutics more efficiently to the tumor sites, reduce the toxicity
during circulation, and improve patient comfort.\textsuperscript{41-43} This development of \textit{nanomedicine}, in fact, is changing the pharmaceutical sciences and industry. Drug-loaded liposomes are the most well-known examples of using nanoparticles as vehicles for drug delivery that are in current clinical use. These bilayered-lipid vesicles are successful examples of carriers for both hydrophilic and hydrophobic agents, delivering small molecular drugs, nucleotides, proteins or imaging agents. Doxil\textsuperscript{®}, for instance, is the commercialized formulation of the anticancer agent, doxorubicin, encapsulated within liposomes, being approved by US Food and Drug Administration (FDA) in 1995 and applied in clinical uses to date. The following paragraphs will depict more examples and recent strategies that have been considered in designing effective therapeutic nanomedicine.

1.2.1 How Size and Shape Matter

A passive strategy for targeting tumors is to design nanocarriers that can accumulate at tumor sites by taking advantage of the enhanced permeability and retention (EPR) effect.\textsuperscript{41,44-46} This phenomenon was identified as an important feature of passive tumor targeting by Matsumura and Maeda in 1986.\textsuperscript{47} The EPR effect is widely believed as the result of the “leaky” endothelium caused by elevated level of angiogenesis (or blood vessels formation) in tumor tissue, which allows the nanoparticles to passively accumulate in the tumor during circulation.

It should be noted that this passive targeting mechanism offered by the EPR effect, is greatly associated with the size and shape of the nanocarriers. Highlighting how the size of the nanoparticles could impact their biodistribution upon circulation, Kotaoka and coworkers have compared the accumulation and effectiveness of different sizes of
long-circulating nanoparticles. They discovered that the drug-loaded nanoparticles could achieve better permeability and enhanced anticancer effect in vivo when the chemotherapeutic nanocarrier was designed to be ~30 nm in diameter. In general, it is considered that the diameter of nanoparticles carrying therapeutics for cancer treatment should be in the range of 10–100 nm, while particles less than 5 nm in their size are usually mostly cleared during circulation via extravasation or renal clearance.

The shape of the nanocarrier is also an important factor. Recent studies indicate the particle shape is comparably important as particle size for both the biodistribution and cellular internalization. In a given example, soft filamentous micelles (filomicelles, with a diameter ~20–60 nm and a length of several microns) are shown to circulate in vivo up to a week, circulating for a much longer periods compared with spherical vesicles injected at the same dose. In this particular example, paclitaxel was loaded in the hydrophobic core of the filamentous micelle. The anticancer drug-loaded filomicelles exhibited prolonged circulation and greater tumor size reduction in animal model than their spherical counterparts, with longer micelles showing increased effectiveness at the same given dose.

1.2.2 Surface Chemistry: Charges, Stealth Properties and Targeting of Nanocarriers

In addition to the geometric factor (size and shape) of the nanocarriers, surface chemistry of the nanoparticles has proven to be a critical feature that will alter the biodistribution in such delivery systems. The surface charge and the surface moiety are two important characteristics that would impact the biodistribution of the nanoparticles when injected intravenously. An important criterion of effective delivery is to achieve
optimal accumulation in the tumorous tissues, a process aided by prolonged circulation. This requires that the carrier must be stable enough to circulate and accumulate in the tumor before being recognized and cleared by the immune system or filtered out by the liver or kidney. Modification of the nanocarrier’s surface chemistry often serves as an effective means to enhance the circulation period after administration.

First, the surface charge of the carriers would strongly affect the stability of the structure during circulation in blood stream, owing to the presence of various proteins in the plasma. It is generally discovered that while positively charged nanocarriers possess a higher nonspecific cellular uptake rate because of the stronger interactions between the carriers and negatively charged cell membranes, it is at the expense of a shorter blood circulation half-life due to the higher tendency to aggregate with the negatively charged proteins in the bloodstream. Neutral or negatively charged carriers, on the contrary, usually have reduced plasma protein adsorption and low rate of nonspecific cellular uptake.

Second, it is crucial to achieve effective therapeutic effect by designing drug-loaded particles that can escape from the immune system before they can perform their function. The stealth properties of therapeutic nano-objects often determine the effectiveness and whether these carriers can accumulate in the tumor. Pegylation at the surface of nanoparticles is a popular method of incorporating stealth properties and achieving prolonged circulation in vivo. Pegylation here refers to the conjugation of polyethylene glycol (PEG), a polyester that has a repeating chemical structure of H-(OCH2CH2)n-OH. PEG is a hydrophilic polymer with many applications in tissue
engineering, regenerative medicine, and drug delivery because of its high biocompatibility. Grafting of PEG on the surface of self-assembled carriers can form a corona that shields the hydrophobic moieties of the swollen polymer brushes, providing steric stabilization and preventing aggregation of the carrier with other plasma proteins during circulation. Pegylated nanoparticles can also allow the carrier vehicles to reduce their rates of clearance by immune cells during circulation by mimicking cells’ glycocalyx that delay immune clearance, thus providing longer-acting effect to carry drug and prolong the therapeutic window after administration. Another interesting example of the stealth feature is to modify the surface of nanoparticles by “self” peptides reported by Discher lab. In this instance, a self peptide was designed based on hCD47, a membrane protein reported as “marker of self” that reduces phagocytosis through signaling between hCD47 and CD172a. This synthesized self peptide serves as a ligand that reduces the clearance by macrophages. Conjugation of this self peptide onto the nanoparticle surface can delay the clearance by phagocytes during circulation, further promoting the accumulation of a therapeutic drug within tumors in vivo.

Conjugation of targeting ligands at the surface of carriers also serves as an effective strategy to actively target the tumor cells for designing effective nanomedicine. Candidates for targeting ligands include antibodies, antibody fragments, proteins, peptide, small molecules, or aptamers, where these ligands introduce highly specific interactions with certain substrates shared by malignant cells or tissues. Surface modification of targeting ligands can enhance the accumulation of nanoparticles, which is associated with receptor-mediated endocytosis of the carriers. In most malignant
tumors, $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins are highly expressed in the tumor endothelium.$^{67}$ Incorporation of integrin-binding RGD (arginine-glycine-aspartic acid) motif into the design of nanomedicines, therefore, also becomes an effective means for targeted delivery of diagnostic probes to tumors. Using RGD peptides for targeting was also applied in designing nanoparticles for drug delivery, and exhibiting beneficial effects to suppress tumor growth or metastasis.$^{68-71}$ Folic acid is another probe commonly used to target the folate receptor, the ligand that is overexpressed in many tumors (cell surface/or endothelial cells).$^{72, 73}$ Though it is still elusive whether folate receptors are mostly overexpressed on cancerous cells or the epithelial cells in tumor vasculature, there are examples showing that drug-loaded nanoparticles can achieve an enhanced therapeutic effect when the particle surface were modified with folic acid as the targeting ligand, offering a mechanism to enhance accumulation and potentially carry therapeutics across the tumor cell membrane.$^{74, 75}$

1.2.3 Self-Delivering Cargos of Therapeutics

As various chemotherapeutics are relative hydrophobic, direct conjugation of hydrophobic agents to a hydrophilic blocks proves to be an effective means to construct self-assembled nano-objects formed by therapeutics, launching the development of self-delivering nanomedicine. Drug-polymer conjugates are molecules where the drug is covalently linked with polymers, serving as emerging candidates for cancer therapy.$^{40, 76}$ There are several examples of anticancer drug-polymer conjugates in clinical development, including paclitaxel, camptothecin, doxorubicin, carboplatin and platinates. Some reported examples includes conjugation of the anticancer drug to
PEG,\textsuperscript{76, 77} poly(L-glutamic acid),\textsuperscript{78} N-(2-hydroxypropyl)methacrylamide (HPMA),\textsuperscript{79, 80} or poly(lactide-co-glycolide) (PLGA) copolymers.\textsuperscript{81} The basic design rationale of these drug-polymer conjugates is mainly aimed to limit the cellular uptake by non-specific endocytosis during circulation and produce longer-circulating materials.\textsuperscript{40} The follow-up benefit of prolonged circulation, as mentioned earlier in section 1.2.2, lies in the promotion of nanocarrier accumulation in tumor sites by the EPR effect instead of non-specific clearance. There are also some instances that demonstrate the ability of tumor targeting by drug-polymer conjugates by incorporating targeting ligands into the design, which can further increase the accumulation of these therapeutics by receptor-mediated delivery.\textsuperscript{74, 75}

In addition to the example of drug-polymer conjugates, the design and synthesis of \textit{drug amphiphiles} initiate another successful platform of using therapeutic to construct self-delivering therapeutics.\textsuperscript{82-84} Drug amphiphiles are amphiphilic molecules where a relatively hydrophobic drug is conjugated with a relatively hydrophilic peptide segment. The drug release profile can be altered by using a degradable or a non-degradable linker between the two moieties. Due to the overall amphiphilic nature, the drug amphiphile can assemble into well-defined nanostructures with a fixed drug loading ranging up to 38\%.\textsuperscript{82} The resulting morphology, in this case, is strongly dependent on the designed peptide sequence. In a given example, the camptothecin-containing drug amphiphile can assemble into nanofibers or nanotubes with a diameter of \(~10\text{ nm}\) by conjugating the anticancer drug to a beta-sheet adopting sequence, such as VQIVYK or GNNQQNY.\textsuperscript{82, 83} These discoveries launch a promising platform to construct
different types of supramolecular polymers composed of anticancer therapeutics with a fixed drug loading.

1.3 Designing Synthetic Materials For Mimicking the Tumor Microenvironments

1.3.1 Mimicking the Tumor Microenvironments

Capturing the behaviors of malignant cells in tumors remains one of the main highlights of oncology, serving as crucial clues to understand tumor progression and seek improvements for the treatments on patients. Migration of cells from a primary location to secondary sites, for instance, is one of the critical steps in tumor progression and metastasis. During the migration process, cells actively remodel and interact with their surrounding extracellular matrices (ECMs). This biological occurrence is highly complex, multistaged and dynamic, being involved with multiple factors that include chemical signals, cell-matrix interactions, and cell-cell interactions.\(^{85}\) Progression of tumor, as much complexity is involved, remains an unsolved subject while many underlying mechanisms are still open to question.

The active role of malignant cells in remodeling their surroundings is relevant to cancer metastasis, the final outcome where all steps in the metastatic cascade have been accomplished by the malignant cells, including 1) the escape of malignant cells from primary sites, 2) extravasation into blood vessels, 3) circulating in the blood stream, 4) extravasation into a secondary tissue, and 5) colonize a second organ.\(^{85}\) It should be noted that every step in this progression is highly characterized by the cell-matrix interactions within the three-dimensional (3D) matrix. A need for 3D synthetic materials,
1.3.2 How Synthetic Materials Connect to Understanding of Tumor Progression

3D synthetic scaffolds were developed due to the demands of using a clean source to accurately capture the complexity of cell behaviors in vitro. Though 2D cell culture has been widely used in routine biological evaluations, most living cells exist in 3D matrices and their behaviors in 2D systems often fail to reflect their functions in vivo. Therefore, synthetic 3D scaffolds are more likely to offer environmental similarity in hierarchical tissues where the cells locate in situ. Additionally since the progression of tumor involves the dynamic interactions between malignant cells and their surroundings, the designed materials must possess a certain responsiveness toward cells in order to ascertain the interactive role of cancer cells. Accordingly, incorporation of the cell-responsiveness into synthetic materials has then been considered as a powerful strategy to recapture the behaviors of cell in natural 3D matrices.

In order to incorporate the cell-responsiveness into the synthetic materials, designing matrices that can be degraded by matrix-metalloproteinases (MMPs) has been shown to be an effective strategy to capture the invasiveness of cells. MMPs are a family of secreted or membrane-bound enzymes (more precisely, proteinases) that mainly play a role in degrading components in ECM, accounting for remodeling of tissue microenvironments, organ development, regulation of inflammatory processes, and acting as crucial regulators in stem cell differentiation, tumor progression and metastasis (Figure 1-4). Since the main functions of MMPs involves the degradation
of the components in ECMS, the overexpression of MMPs are strongly associated with the enhanced cellular invasiveness, an important factor that explains why tumor cells can escape from the primary tumor and further spread into another organ during metastasis. Here we would like to introduce some advances and examples of incorporating MMP-degradability into synthetic materials, including both polymeric hydrogels and supramolecular hydrogels.

![Figure 1-4. The multifaceted role of MMPs in tissue invasion, angiogenesis, regulation of inflammation and metastatic niche. Adapted from ref (98).](image)

1.3.3 Incorporation of MMP-Degradable Features into Polymeric Hydrogels

Hubbell and coworkers reported examples of synthetic hydrogels that captures the cellular invasiveness. They initially selected a family of peptide sequences that were susceptibly degraded toward MMP-1 and MMP-2. By using an MMP-specific substrate to crosslink low-molecular-weight PEG, the resulting polymeric hydrogels
have shown a decrease in matrix stiffness when treated with MMPs, attributed to the specific breakdown of crosslinking peptides (Figure 1-5a). The developed methodology provides a model for engineering hydrogels that capture the cell invasiveness. In addition, these responsive hydrogels may also be useful in tissue engineering as alternatives for naturally occurring ECM-derived materials.89, 91

1.3.4 Engineering MMP-Degradability into Supramolecular Filament Networks

As mentioned in section 1.1, self-assembling peptides are powerful building blocks to construct supramolecular polymers via non-covalent interactions in aqueous media. Molecules that exhibit strong anisotropic interactions tend to assemble into 1D supramolecular structures; at higher concentration, the resultant filaments (whose width is usually at nano scale) can often entangle into 3D networks and provide robust mechanical properties to support the cells as scaffolds.

Successful instances of utilizing bottom-up approach to construct MMP-degradable filament networks include the multidomain peptide hydrogel reported by Hartgerink lab,100 self-assembling peptides with MMP-2 hexapeptidic substrate reported by Langer lab,101 and the self-assembled hairpin peptide reported by Schneider and coworkers.102 In these given examples, MMP degradable sequences are selected and designed within the amphiphilic peptides that strongly adopt β-strand conformation (Figure 1-5b). These designed peptides self-assemble into filament networks, and form hydrogels in aqueous media. Upon the specific cleavage of the peptidic substrate by MMP, the networks would dissociate, which serves as the ECM-mimicking materials that capture the breakdown process by proteinases.
Figure 1-5. Reported strategies of the incorporation of MMP degradable features into synthetic hydrogels as modeling materials for engineering cell invasiveness. a) The use of a MMP degradable sequence to crosslink PEG allows the resulting hydrogels be specifically degraded upon the treatment of MMP. b) Construction of self-assembled filamentous networks via direct incorporation of MMP-13 specifically degradable peptide sequence into β-turn peptide that supramolecular polymers. Adapted from ref (99) and ref (102).

1.4 Thesis Overview

Functional supramolecular polymers provide a platform to fabricate materials with applications in multiple disciplines. This platform offers great potential in overcoming obstacles and achieving particular functions through the rational design of building blocks. In-depth understanding of the connections between monomers and supramolecular behaviors will contribute to better control over the characteristics of the resulting materials when designing effective systems for biological or biomedical applications. The following chapters will cover several examples of the construction of different types of 1D structures via self-assembly using synthetic peptides/peptide conjugates as building blocks. Chapter 2 and Chapter 3 highlight the use of drug-peptide conjugates (drug amphiphiles) to fabricate nanotubes by altering the molecular
packing via two distinctive approaches. Chapter 4 introduces a unique miktoarm star construct when designing amphiphilic peptides to alter the morphology of self-assembled structures. Chapter 5 and 6 continue the miktoarm star construct mentioned in the previous chapter, and serve as an initial attempt to fabricate filamentous networks with controlled enzymatic degradability and mechanical properties.
2 Construction of Anticancer Drug Amphiphiles and Their Morphological Behaviors in Catanionic Mixtures*

2.1 Abstract

Mixing of oppositely charged amphiphilic molecules (catanionic mixing) offers an attractive strategy to produce new morphologies different from those formed by individual molecules. We report here on the use of catanionic mixing of anticancer drug amphiphiles to construct multi-walled nanotubes containing a fixed and high drug loading. Notably, cryogenic transmission microscopy reveals that these self-assembled nanotubes display certain degrees of internal fluidity, in contrast to the rigid filaments formed by each individual drug amphiphile under the same conditions. We found that the molecular mixing ratio, the solvent composition, the overall drug concentrations, as well as the molecular design of the studied amphiphiles are all important experimental parameters contributing to the tubular morphology. We believe these results demonstrate the remarkable potential that anticancer drugs could offer to self-assemble into discrete nanostructures, and also provide new insight into the formation mechanism of nanotubes by catanonic mixtures.

2.2 Introduction

Nanotubes are one-dimensional (1D) hollow nanostructures that could possess dimensionality-related electronic, mechanical, or biological properties, and have attracted extensive research interest over the past two decades due to their potential applications in electronics, catalysis, and drug delivery. From the manufacturing perspective, nanotubes can be produced via vapor deposition techniques (e.g. carbon nanotubes, diphenylalanine nanotubes), by using high-aspect-ratio nanostructures as precursors or templates, or through solution state molecular self-assembly—a facile process that typically does not require high temperature and lower pressure, or involve deposition and etching procedures. A number of molecular building units have been shown capable of spontaneously associating into a tubular morphology upon dissolution into a selective solvent, including block copolymers, lipids, cholesterol derivatives, peptides. Of many molecular features appearing to contribute to formation of the tubular morphologies composed of bilayer structures, the ability to pack anisotropically in a highly ordered fashion within two dimensions seems to be a general requirement for most molecular building blocks. As a consequence, compared to the canonical spherical, cylindrical, and vesicular morphologies, tubular structures are rarely observed in most self-assembling systems. In this communication, we report the first use of anticancer drugs as molecular building blocks to construct multi-walled nanotubes. Notably, the nanotubes reported here show great bending flexibility and represent the first example of its kind.
Self-assembly of therapeutic agents into well-defined nanoscale objects offers an innovative strategy to construct self-delivering supramolecular nanomedicine with controlled pharmacokinetic properties. Since a majority of anticancer drugs do not carry the amphiphilic feature necessary for self-assembling into discrete nanostructures in aqueous environments, an important step in this strategy is the design and construction of amphiphilic drug molecules through conjugation with another hydrophilic chemical moiety. The resultant amphiphilic conjugates could potentially serve as effective building blocks to construct versatile nanostructures of various sizes and shapes. However, unlike the traditional carrier-based drug delivery strategies where the carriers can be constructed from a great diversity of molecular building units, the use of a drug to build nanocarriers of its own is limited by the molecular design and synthetic feasibility. It is therefore necessary to seek other assembly strategies to access different morphologies required for controlled delivery properties in various conditions.

Co-assembly of two or more different amphiphilic molecules is one such strategy that can be used to influence both the functional and structural properties of the nanostructures formed. Mixing of amphiphiles with opposing charges, for example, provides a means for controlling the surface charge of the assembly through variation of the mixing ratio and can be used to introduce multiple functionalities if the two amphiphiles being co-assembled have differing epitopes. These catanionic mixtures (CAMs) can also possess intriguing phase behaviors and usually offer alternative morphologies compared with that formed by each individual amphiphile. It is generally believed that the formation of ion pairs by oppositely charged surfactants reduces the
electrostatic repulsions among the headgroups, leading to a reduced curvature to form bilayered supramolecular nanostructures. This strategy of mixing two oppositely charged amphiphilic molecules has been used to produce a variety of bilayered nanostructures, including vesicles, icosahedra, nanodiscs, and nanobelts. Tubular structures, which can be considered as curved bilayer architectures, are rarely reported in catanionic systems. We show here that catanionic mixtures of two drug amphiphiles (DAs)—each with a 36% fixed loading of the anticancer drug camptothecin (CPT)—lead to formation of a multi-walled nanotube morphology that also possesses a 36% fixed CPT loading.

The DAs used in this study were designed to contain either one, two or four hydrophobic CPTs that are conjugated to a β-sheet forming peptide sequence through a reducible disulfylbutyrate (buSS) linker (Figure 2-1a and Scheme S2-1 in SI).82 The peptide segments with N-terminal cysteine residues were first synthesized using standard Fmoc-solid phase peptide synthesis protocols. The conjugation of the peptide with the CPT units was carried out in DMSO, utilizing a direct disulfide formation approach in which the peptide cysteine(s) displace thiopyridinone from CPT-buSS-Pyr. The peptide moiety GNNQQNY is a key β-sheet forming sequence derived from the yeast prion Sup35,103 and was chosen to afford the capacity for intermolecular hydrogen bonding between the designed DAs. Two lysine (K) or two glutamic acid (E) residues were placed at the C-terminal of the studied peptide to imbue both the overall amphiphilicity and the charge status (positive or negative). The main DA of interest, qCPT-Sup35, possesses four CPTs that are chemically bonded to the peptide moiety,
giving a fixed drug loading of 36% (See SI S1). Both the C-terminal K and E DAs were synthesized, furnishing qCPT-Sup35-K₂ and qCPT-Sup35-E₂, respectively.

Figure 2-1. Self-assembly behaviors of qCPT-Sup35-K₂, qCPT-Sup35-E₂ and the CAM formed by the two drug amphiphiles. a) Chemical structures and schematic representations of qCPT-Sup35-K₂ and qCPT-Sup35-E₂. b–c) Schematic representations and TEM micrographs of the supramolecular structures formed by qCPT-Sup35-K₂, qCPT-Sup35-E₂, and the CAM of the two DAs. Both qCPT-Sup35-K₂ (b) and qCPT-Sup35-E₂ (c) formed single filaments in 1:1 MeCN/H₂O after the materials had been previously treated with HFIP. The widths of the single filaments measured from TEM are 5.7 ± 0.9 nm, and 5.9 ± 0.9 nm for qCPT-Sup35-K₂ and qCPT-Sup35-E₂, respectively. d) The CAM of qCPT-Sup35 (mixing ratio 1:3) results in the almost exclusive formation of tubular structures in 1:1 MeCN/H₂O. The tubular size measured from cryo-TEM imaging is 123 ± 28 nm. Total concentration for all samples = 400 μM.
2.3 Experimental Procedures

2.3.1 Synthesis

*Peptide Synthesis* The peptides utilized in this study were synthesized employing standard Fmoc solid-phase protocols, using either Rink Amide MBHA or Fmoc-Glu(tBu)-Wang resins at a 0.25 mmol synthesis scale. An automated peptide synthesizer (Focus XC, AAPPTec, Louisville, KY) was employed to build the Sup35 sequence, before manual protocols were used to furnish the branching motif. Fmoc deprotection of the Fmoc-amino acids was established by treating the resin with 4-methylpiperidine in dimethylformamide (DMF) (20% v/v). The amino acid coupling cycle was performed after Fmoc-deprotection, and the resin was treated with Fmoc-amino acids, O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), and diisopropylethylamine (DIEA) (4:4:6 mole equiv to resin) in DMF for 1 h. The branching molecular design was achieved by coupling Fmoc- Lys(Fmoc)-OH at the N-terminus to yield multiple primary amine groups after Fmoc-deprotection for the further coupling of Fmoc-Cys(Trt)-OH in a branch construct. The N-termini of the peptides were acetylated with 20% acetic anhydride in DMF. Peptides were then cleavage from the resin by trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/1,2-ethanedithiol (EDT)/ H2O (92.2:2.5:2.5:2.5) for 2 hr. The liquid residue was concentrated *in vacuo*, and added into ice-cold Et2O to yield pale precipitates. These crude peptides were then filtered, collected, kept in the desiccator to remove the remaining solvent, and were directly used for the following reaction.
4-(pyridine-2-yl disulfanyl)butanoic acid This was synthesized by a modified protocol based on previously reported work. In brief, 4-bromobutyric acid (2.0 g, 12.0 mmol) and thiourea (1.06 g, 14.0 mmol) were refluxed in ethanol (50 mL) for 4 h. After adding NaOH (4.85 g, in 10 mL of EtOH) into this solution, the mixtures were refluxed for an additional 16 hr. After cooling the solution to room temperature, the solution was concentrated in vacuo, and diluted with water that was extracted twice with ethyl ether. The aqueous phase was then acidified by 4 M HCl to pH 5, resulting in a cloudy solution that was extracted by ethyl ether. The organic portion was dried with Na₂SO₄ and concentrated to yield clear, oil-like 4-sulfanylbutyric acid. 4-sulfanylbutyric acid was then dissolved in MeOH and added dropwise into a 5-mL methanolic solution of 2-aldrithiol (3.03 g, 0.0137 mol). The mixtures were allowed to react for 3 h and were purified by RP-HPLC. Purified products were collected and solvents removed in vacuo. The resultant yellowish oil was then dissolved in CHCl₃, dried over Na₂SO₄, and solvent removed to give HO₂C-buSS-Pyr as a light yellow oil.

Camptothecin-4-(pyridin-2-yl disulfanyl)butanoate (CPT-buSS-Pyr) Camptothecin (CPT, 200 mg) and dimethyl- aminopyridine (DMAP, 44 mg) were initially suspended in DCM (32 mL). HO₂C-buSS-Pyr (280 mg) and diisopropylcarbodiimide (DIC, 436 μL) were then added to the mixtures and stirred for 36 h. This solution was then filtered, diluted with chloroform (60 mL), washed with NaHCO₃ (50 mL) and brine (50 mL), then dried over Na₂SO₄ and concentrated in vacuo. The crude product was purified by flash chromatography using EtOAc (500 mL) and then 0.5% MeOH in EtOAc (250 mL).
Collected products were identified by TLC and underwent solvent removal *in vacuo* to yield a yellow solid.

*Syntheses of the DAs and Purification* The syntheses of all DAs were carried out by dissolving **CPT-buSS-Pyr** and the corresponding crude peptides in N₂-purged dimethylsulfoxide (DMSO, 1 mL) (2:1 by mol). The mixtures were allowed to react for 5 d at room temperature, and the resulting solutions were diluted by MeCN/H₂O, and then purified by RP-HPLC. Eluted products after purification were collected, flash frozen by liquid nitrogen, and lyophilized. The purity of the collected products were confirmed by RP-HPLC and ESI-MS, and the conjugate concentrations were calibrated before aliquotting into predetermined amounts.

**2.3.2 Calibration of the Concentration**

The concentration of DAs was calibrated by analyzing the reduced product from the DAs — **CPT-buSH**. A stock solution of the DA was prepared by dissolving in MeCN/H₂O (1:1). 5 μL of the stock DA solution was then diluted to 20 μL by MeCN/H₂O (1:1), and mixed with 20 μL of 1 M 3,3',3''-phosphanetriyltripropanoic acid (TCEP) for 45 min. The resultant solution was then analyzed by RP-HPLC, monitoring the absorbance at 370 nm. The CPT concentration of the analyte solution was then calculated from the area under the curve in the chromatogram by comparing with the standard solutions based on the content of **CPT-buSH**. The conjugate concentration of the stock solution was then determined based on the applied dilution and the number of CPT molecules.
2.3.3 Preparation of the Catanonic Mixtures (CAMs)

The two components of the CAMs were dissolved in hexafluoroisopropanol (HFIP) separately to yield clear solutions. The CAMs were prepared by mixing the two DAs that were dissolved in HFIP, with mixing ratios of 1:3, 1:1 or 3:1 by charge, whilst the total peptide amount was kept constant in each vial (by mole). The mixtures were then flash frozen in liquid nitrogen and lyophilized to yield dry solids. Solutions were then reconstituted in 1:1 MeCN/H₂O and aged overnight for further studies.

2.3.4 Transmission Electron Microscopy (TEM)

The TEM samples were prepared by negative staining techniques. The solutions (7 μL) were initially loaded on copper grids covered with carbon film. The liquids were then removed by filter papers, and 2% uranyl acetate solutions (7 μL) were subsequently deposited on the samples. The uranyl solutions were then removed by filter papers after 30 s. The specimens were air-dried prior to imaging. Bright-field TEM imaging was performed on an FEI Tecnai 12 TWIN electron microscope at 100 kV. The micrographs were acquired by a SIS Megaview III wide angle camera.

2.3.5 Cryogenic-Transmission Electron Microscopy (Cryo-TEM)

Specimens of cryo-TEM imaging were prepared by Vitrobot (FEI, Hillsboro, OR). The solutions were initially loaded on a copper grid with Lacey carbon film (Electron Microscopy Sciences, Hatfield, PA) in the controlled humidity chamber, and were blotted by the filter papers that were mounted on the Vitrobot from the both sides of the grid. This process engenders a thin film of solutions that adhere on the sample grid. The blotted samples were then transferred into liquid ethane, and were stored in liquid
nitrogen until further use. Sample imaging was conducted on a FEI Tecnai 12 TWIN electron microscope at 100 kV. The micrographs were acquired by a 16 bit 2K×2K FEI Eagle bottom mount camera.

2.3.6 Circular Dichroism Spectroscopy

The CD spectra were monitored by using a Jasco J-710 spectropolarimeter (JASCO, Easton, MD). Solutions were loaded in 1 mm cuvettes and the spectra were recorded in the far-UV and Vis region. The molar ellipticity was calculated by the following equation

$$[\theta] = \frac{\theta}{lC}$$

where $[\theta]$ represents the molar ellipticity, $\theta$ is the measured ellipticity in deg, $C$ is the concentration of the DAs in dmol•cm$^{-3}$, and $l$ is the light path length of the cuvette in cm.

2.3.7 Zeta Potential Measurements

The zeta-potential measurements were performed on a Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK). The prepared solutions were loaded in folded capillary cells and equilibrated for 2 min prior to measurements. The zeta- potential of the assembled structures was obtained by measuring the electrophoretic movement of the nanostructures under the applied electric field, where the movement velocity is determined by phase analysis light scattering.
2.4 Results and Discussions

To ensure molecular level mixing of DAs and to preclude possible pre-assembled structures, the CAMs were prepared using the following procedure: first, each molecule was dissolved in hexafluoroisopropanol (HFIP)—a solvent that is disruptive toward the formation of assembled structures for all the studied molecules. Next, two solutions containing the respective molecules were mixed at a desired molar ratio and the mixed solution was then lyophilized. The lyophilized material was dissolved in 1:1 MeCN/H\textsubscript{2}O to a desired concentration and aged overnight, followed by cryogenic transmission microscopy (cryo-TEM) imaging. Cryo-TEM enables the direct visualization of the assembled structures in vitrified water, minimizing artifacts that can result from the drying and staining of samples in conventional TEM.\textsuperscript{104}

At 400 μM, the CAMs formed by \textbf{qCPT-Sup35} revealed dominant tubular structures when mixed at a 1:3 ratio of amine to carboxylic acid (1:3 for the following notations) in 1:1 MeCN/H\textsubscript{2}O after aging overnight (Figure 2-1d). The two-dimensional projection of a tubular structure in cryo-TEM imaging differs from other types of self-assembled construct, showing two parallel, darker lines at the edges and a shady span in the tubular body. This contrast can be attributed to the high electron density of the densely packed hydrophobic core and the relatively longer traveling distances of the electrons through the nanotube edges. In contrast, the individual DAs were found to assemble into filamentous nanostructures (~6 nm in diameter in both cases), when reconstituted in 1:1 MeCN/H\textsubscript{2}O using the exact HFIP-pretreatment protocols (Figure 2-1b–c; further discussions of the supramolecular structures formed by the individual DA are provided
This clearly suggests that it is the mixing of the cationic and anionic forms of qCPT-Sup35 that gives rise to the tubular morphology.

These observed tubular structures in CAM solutions possess outer diameters of ~123 nm and wall thickness up to ~25 nm (measured from cryo-TEM micrographs). Assuming the qCPT-Sup35 CAMs form a single bilayer, the maximum shell thickness would be comparable to twice the extended length of the individual DAs—approximately ~10 nm. Thus, the greater wall thickness observed in cryo-TEM micrographs (~25 nm vs. ~10 nm) suggests the presence of multiple bilayers in some assembled structures. Indeed, multi-walled constructs could occasionally be observed in cryo-TEM imaging (Figure 2-2a–b), providing direct support for this inference.

Figure 2-2. TEM and cryo-TEM micrographs of CAM of qCPT-Sup35 with a mixing ratio of 1:3 at 400 μM in 1:1 MeCN/H₂O. a–b) Bending of the tubular structures (indicated by the red arrows) and the direct observation of the multi-walled nature of the tubules (labeled with blue arrows) in cryo-TEM imaging. c–d) Undulation of the tube widths is commonly observed in regions where overlapping occurs. All bars = 200 nm.
Another interesting observation of these nanotubes in cryo-TEM imaging is their buckling nature that is very similar to membrane compression. Occasionally, it could be spotted that the otherwise straight nanotubes were bending, appearing as a “folded straw” structure in which pinching of the tube was seen at the bend point (Figure 2-2a–c). This bending did not lead to the breakdown of the tubular structures unless the angle of bending exceeded 90°, and even so the two sections still remained connected (Figure 2-2b). In addition, we also observed irregular undulations in the tube diameter (Figure 2-2c–d and Figure S2-9a). The broadening of the tubular diameter did not display any periodic pitch and generally occurred in regions where the tubules overlapped with other nanotubes or with the lacey carbon film. We therefore speculate that this “squeezing” of the tubules may be a result of being confined into the thin vitreous water layer (typically less than ~250 nm) that were generated in the cryo-TEM sample preparation. Both observations imply that molecular packing within these tubules are reminiscent of that of fluidic-like bilayers. This is in sharp contrast with reported tubular morphologies that are often rigid and have a well-ordered internal packing.\textsuperscript{105, 106}

We found that the overall mixing ratio plays a critical role in defining the final assembled morphologies. Dominant tubular structures were only observed for mixing ratios of 1:3–1:5 (amine to carboxylic acid) at the concentration of 400 μM. Zeta potential measurements reveal that these nanotubes remain almost neutral on their surface (−3.79 ± 2.86 mV for \textit{qCPT-Sup35 CAM} 1:3) (Figure 2-3c); this may imply most of the amphiphiles formed ion pairs so that the charges were neutralized. Increasing the amount of \textit{qCPT-Sup35-K}: in the mixture to give a 1:1 ratio of amine to carboxylic acid,
however, led to the coexistence of tubules and filamentous structures (Figure 2-3a; zeta potential: 8.68 ± 3.79 mV). As the content of qCPT-Sup35-K2 was raised to yield a mixing ratio of 3:1, filamentous structures started to dominate; large tubules could still be observed, but displayed a significant decrease in length (on the order of a few µm) (Figure 2-3b and Figure S2-9f).

![Figure 2-3. Characterizations of CAMs of qCPT-Sup35 at different mixing ratios. a–b) Cryo-TEM micrographs of qCPT-Sup35 CAM with a mixing ratio of 1:1 (a), and 3:1 (b) at 400 µM in 1:1 MeCN/H2O. All bars = 500 nm. c) Zeta potential measurements of the qCPT-Sup35 CAMs with different mixing ratios. Data are presented as mean ± s.d. d) CD spectra of the qCPT-Sup35 CAMs 1:3, and individual DAs in 1:1 MeCN/H2O. All spectra were obtained from 400 µM solutions that were diluted to 50 µM immediately prior to measurement.]

Circular dichroism (CD) spectroscopy provides additional insight into the secondary structure of the peptide domain of the DAs, and also into the environment of the CPT moieties. Analysis of the 1:3 qCPT-Sup35 CAM in 1:1 MeCN/H2O reveals a number of interesting signals (Figure 2-3d). First, a strong negative peak at 215 nm can
be seen, a characteristic indicator of the β-sheet conformation that is in agreement with the one-dimensionality of the assembled structures. Second, signals from the CPT moieties are also evident, displaying a negative signal at 250 nm (n–π*), and a series of signals between 330 nm and 500 nm (π–π*) that are caused by the chiral packing of CPT moieties in the assembled form. Third, the lack of any truly bisignate peaks suggests that the CPT molecules may be aligned in a parallel fashion, rather than with a helical sense as was previously observed in the Tau peptide analogue. Comparison with the CD spectra of the individual DAs under the same assembly conditions shows there are some similarities in the peptide absorption region with the major difference in the CPT absorption regions—the tubes having a very broad negative signal between 400 and 500 nm (Figure 2-3d). The physical origin of this peak is unclear, but may be linked to the formation of the nanotubes, given the fact that this peak also appears in the CD spectra of CAMs with different mixing ratios that exhibit the tubular structures (Figure S2-10). These results clearly suggest that it is the arrangement of CPT units that is primarily responsible for the formation of the tubular morphology.

Observations of intermediate structures often provide evidence for the mechanistic pathway and kinetic processes that engender the formation of large structures like nanotubes. This information can be obtained by variation of assembly conditions such as concentration and solvents. We found that the dominant supramolecular morphology for qCPT-Sup35 CAM 1:3 is strongly dependent upon two factors—the bulk concentration and the MeCN:H2O ratio. In the case of the former, we found that intermediate structures could be seen at lower concentrations in 50% MeCN (Figure 2-
4a–d). At 10 µM, clusters of belt-like structures were more commonly observed (Figure 2-4a–b). As the bulk concentration was increased to 50 or 100 µM, more complex assemblies such as helical ribbons (Figure 2-4c) and ribbon-wrapped tubes appeared (Figure 2-4d). The pitch angle of these helical ribbons is consistently close to 45°, a feature predicted theoretically in curved membranes that form helical ribbons or tubules characterized by chiral packing. In the Helfrich-Prost model for the assembled layers possessing isotropic rigidity, this tilt angle is a result of the minimization the elastic free energy for membranes with a cylindrical curvature. In addition, the ribbon-wrapped tubes provide direct evidence for the multi-walled nature of these tubules, accounting for the tubular thickness variation observed at higher concentrations. It is noted that the observed ribbons display attenuating widths gradually at the ends with a fixed pitch, implying that formation of the tubules results from widening of ribbons rather than from the shrinking of helical coils (Figure 2-4b).

Variation of the solvent composition was found to have a similar effect on the self-assembly of qCPT-Sup35 CAMs 1:3. It was found that the CAMs could not be directly reconstituted in pure water after the HFIP treatment, instead suffering from low material solubility that did not lead to the formation of any well-defined structures. Similarly, the qCPT-Sup35 CAMs also exhibited poor solubility in pure MeCN, immediately forming huge precipitates with no well-defined structures observed by TEM. Though a qCPT-Sup35 CAM gave a turbid solution when reconstituted in 3:1 MeCN/H2O at 400 µM, intermediate structures could still be observed in TEM imaging, exhibiting similar multilayered helical ribbon and ribbon-wrapped tubule structures to
those discovered at 50 or 100 μM when using 1:1 MeCN/H₂O as solvent (Figure 2-4e–4f).

Again, both types of intermediate structures are indicative of at least two independent growth pathways for the studied system: multilayer formation and tubular formation from helical ribbons.

![TEM micrographs of the intermediate structures of CAM of qCPT-Sup35 (1:3) in 1:1 MeCN/H₂O, unless stated otherwise.](image)

Figure 2-4. TEM micrographs of the intermediate structures of CAM of qCPT-Sup35 (1:3) in 1:1 MeCN/H₂O, unless stated otherwise. a–b) Clusters of belts and helical ribbons are the dominant structures at 10 μM (a), and 50 μM (b). c) A multi-layer helical ribbon at 50 μM. d) An intermediate multi-wall tubular structure in a 100 μM solution, showing a tubular structure wrapped with another layer of belt. e–f) Similar intermediate structures, such as multilayer helical ribbons and intermediate multi-wall tubular structures in 3:1 MeCN/H₂O, where the materials exhibited limited solubility. Bulk concentration = 400 μM.

Since the catanionic mixing of amphiphiles has been reported to generate a variety of possible morphology, one important parameter yet to be considered in our system is the intrinsic packing tendency of the studied drug amphiphiles. We therefore
prepared CAMs for mCPT-Sup35 and dCPT-Sup35 possessing one and two CPT molecules, respectively, under exactly the same conditions to produce qCPT-Sup35 nanotubes. In the case of catanionic mixing of mCPT-Sup35-K2 and mCPT-Sup35-E2 in 1:1 MeCN/H2O at 400 µM, well-defined structures were only scarcely observed. The CAM of dCPT-Sup35 exhibited exclusively 1D filamentous structures in 1:1 MeCN/H2O at 400 µM regardless of the mixing composition tested (mixing ratio of amine to carboxylic acid: 1:3, 1:1, or 3:1, see Figure S2-11 in SI), similar to those observed for the individual DAs of qCPT-Sup35. CD spectra of dCPT-Sup35 CAM, dCPT-Sup35-K2, dCPT-Sup35-E2 all display a negative peak at ~215 nm that corresponds to the β-sheet secondary structure and signals from the CPT chromophores in the 260–410 nm range (Figure S2-12 in SI). The fact that few differences in the CD were detected for the CAMs of dCPT-Sup35 when compared with the individual components, suggests there is no change in the packing arrangement of the CPT molecules upon CAM formation, especially considering the occurrence of the negative signal in the 400–500 nm range that was observed for the qCPT-Sup35 CAMs. Consequently, the packing requirements of the hydrophobic core within the assemblies formed by CAMs of qCPT-Sup35 must play a crucial role for the mixtures to adopt tubular structures as their dominant supramolecular morphology.

Our results suggest the molecular mixing ratio, the solvent composition, the overall concentration, as well as the molecular structures of the studied drug amphiphiles are all important experimental factors to define the tubular morphology. On the basis of our observation of the intermediate helical ribbons, the most plausible kinetic pathway to
form these observed multi-walled nanotubes by qCPT-Sup35 CAM is the widening of the helical ribbons that are composed of multiple bilayered structures. The forming process is likely a cumulative result of 1D elongation, multiple bilayer formation, and bilayer extension (Figure 2-5b).

Figure 2-5. Proposed mechanism of the tubular formation by the CAM of qCPT-Sup35. a) Schematic illustration of the effect of packing parameter of qCPT-Sup35 and dCPT-Sup35 on the formation of ion pairs and the resulting supramolecular morphology of corresponding catanionic mixtures. b) The formation of the multi-wall nanotubes by CAMs of qCPT-Sup35 is the cumulative result of three occurrences: 1D elongation, formation of multilayers, and bilayer extension from helical ribbons. The CAM of qCPT-Sup35 formed bilayers, where the direction of lateral bilayer extension is perpendicular to the orientation of the intermolecular hydrogen bonds.

We speculate that both bilayer formation (packing geometry) and chiral stacking (packing directions) play a key role in the formation of nanotubes reported here. First, the importance of the bilayer formation can be directly evidenced by the observations that only qCPT-Sup35 CAMs can form the tubular morphology. Both mCPT-Sup35 and
dCPT-Sup35 CAMs form filamentous nanostructures under the same conditions. It is now well accepted that the geometric shape of amphiphilic molecules strongly affects the resultant supramolecular morphology as a balance of the effective volume of the hydrophobic segment with that of the headgroup. In a catanionic system, it is expected that electrostatic attractions as a result of ion pairing of the oppositely charged headgroups would reduce the effective volume of the headgroup areas, thereby leading to a reduced interfacial curvature. Indeed, several CAM systems suggest that catanionic mixing of sphere or cylinder-forming amphiphiles could produce bi-layered structures. This can also be used to explain reasonably our system. Neither the CAMs of mCPT-Sup35 and dCPT-Sup35, nor individual qCPT-Sup35-K2 and qCPT-Sup35-E2 could satisfy the requirement for a bilayer packing geometry due to either the relatively smaller hydrophobic segments, or the relatively large headgroup areas. The multi-walled construct is reminiscent of multi-lamellar vesicles commonly observed in concentrated catanionic mixtures. This stacking is known to be driven by short-range interactions, such as attractive van der Waals interaction, electrostatic interactions, hydration force, and attractive hydrophobic interactions.

Second, the observation of helical ribbons as the nanotube precursor morphology strongly suggests the formation of tubular structures in our system may, from a supramolecular assembly perspective, share common features with the lateral extension of helical ribbons into tubules of chiral self-assemblies reported by other laboratories. We speculate that the chiral packing in our system stems from the Sup35 peptide segment, but is also affected by the stacking among the CPT units. Peptides are known
to form twisted/curved 1D nanostructures.\textsuperscript{117-119} The formation of intermolecular hydrogen bonding in a chiral manner among the Sup35 peptide can be supported by the typical $\beta$-sheet absorption in the CD spectra (\textbf{Figure 2-3d}). However, given the fact that $\pi-\pi$ interactions among the CPT moieties are also known to lead to 1D chiral structures, it is difficult to deconvolute the contribution of the CPT interaction from hydrogen bonding in our system for the formation of nanotubes.

\textbf{2.5 Conclusions}

In summary, we have successfully utilized the CAMs of therapeutic DAs to form tubular constructs with multi-walled architectures. The resulting tubular assemblies potentially exhibit unique characteristics when compared with rigid nanotubes, one notable example being their greater flexibility. The results of our study reveal that such structures are formed through the combination of 1D elongation and bilayer extension that is induced by a significant reduction in curvature as a result of catanionic mixing and the hydrophobic core’s geometrical requirements. Using these observations as general guidelines, it should be possible to design building blocks for the construction of tubules with other functional properties through the self-assembly of catanionic mixtures.

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3 Designing Stimuli Responsive Drug-Amphiphiles for the Construction of Nanotubes

3.1 Abstract

Amphiphilic siderophores are iron-chelators that are initially found to be secreted by marine bacteria and offer a mechanism to change their supramolecular morphology (such as a micelle-to-vesicle transformation) upon iron chelation, which is owing to the coordination-induced change in packing geometry from a more conical like structure into a less conical like geometry. This unique morphological transformation, though can be classified as a stimuli-responsive instance, is rarely applied in the rational design of synthetic materials in bottom-up strategies to construct smart materials that are responsive toward metal ions. Herein, we report an example of a synthetic, therapeutic, siderophore-based amphiphile that possesses a “filament-to-tube” morphological transformation of the resultant assembled structure upon iron chelation. This observed structural transition in this reported system is analogous to the change of packing geometry when directional interactions involved in the monomers of the supramolecular assemblies. This design rationale is believed to be an effective approach with an aim to construct stimuli-responsive smart materials or powerful means to construct self-assembled nanotubes.
3.2 Introduction

Designing stimuli responsive materials have gained much attention for the development of smart, interactive, and functional materials.\textsuperscript{120-123} Materials that exhibit behavior transition upon metal coordination, for instance, have exhibited diverse applications in constructing nanoelectronic materials, biosensors, or metal ions containing hydrogels.\textsuperscript{123} Amphiphilic siderophores are iron chelators initially isolated from bacteria, possessing unique responsiveness in their self-assembling behaviors upon iron chelation, where the assembled structures can undergo micelle-to-vesicle transition.\textsuperscript{124, 125} This coordination triggered transformation of assembled structures, though can be considered as a stimuli responsive behavior, however, is rarely included in strategies for controlling the supramolecular chemistry for synthetic materials. Despite precedent work that has shown the siderophore conjugates can be applied in drug delivery or medical imaging due to their unique chelating behaviors with metal irons,\textsuperscript{126-128} little was investigated regarding to the aspect of the self-assembling behaviors of these conjugates. Herein, we reported an example of an amphiphilic siderophore-based drug amphiphile (DA) that exhibits morphological transition from nanofilaments into nanotubes upon the iron coordination.

Formation of supramolecular structures characterized by one-dimensional (1D) growth is often governed by anisotropic interactions among the building blocks.\textsuperscript{25, 105} This feature has been considered as a classic criteria when designing small molecules that can spontaneously self-assemble into 1D supramolecular structures, including nanofibers,\textsuperscript{17, 21, 129} nanobelts,\textsuperscript{118} or nanotubes,\textsuperscript{22, 116, 130} by designing molecules that possess
strong directional, associative interactions such as hydrogen bonding\textsuperscript{21, 22, 118} or \(\pi-\pi\) stacking.\textsuperscript{130} We previously reported the self-assembled 1D structures can be constructed through the rational design of DAs, amphiphilic molecules that comprise hydrophobic therapeutics, by incorporating a relatively hydrophilic \(\beta\)-sheet adopting peptide sequence in the molecular structure.\textsuperscript{82, 84} These designed DAs can segregate into 1D nanostructures owing to the strong hydrogen bonds among the monomers, possessing fixed drug loading based of the number of therapeutic agents conjugated on the peptides, and serving as self-delivering nanomedicine in drug-delivery.

In this study, in order to incorporate the responsiveness toward metal chelation, we designed a DA that carries a deferoxamine B (DFO) segment, a hydroxamate containing siderophore isolated from actinobacteria \textit{Streptomyces pilosus}, and camptothecin (CPT), an anticancer therapeutic agent that potentially induces intermolecular \(\pi-\pi\) stacking because of its rich aromatic nature, jointed by a reducible disulfide bond that bridges disulfidylbutyrate (\textit{buSS}) linker and a cysteine residue (\textbf{Figure 3-1a}).
Figure 3-1. Self-assembly behavior of DFO-C-CPT. a) The chemical structure of the designed drug amphiphile DFO-C-CPT and schematic representation of the self-assembling behaviors. DFO-C-CPT assembles into nanofilaments when it is dissolved in water, while it assembles into nanotubes if Fe(III) is present. b–c) TEM micrographs of the filaments formed by DFO-C-CPT in H2O (c) and the nanotubes formed by DFO-C-CPT in 1 equiv Fe(III). d) Cryo-TEM micrograph of the nanotubes (indicated by white arrows) formed by DFO-C-CPT. e) Coordination of Fe(III) triggers the morphological change from filaments to tubes. Specimen of TEM micrograph was prepared by the addition of Fe(III) into the solution after DFO-C-CPT formed filaments in H2O. Regular TEM specimens were stained by 2% (w/v) uranyl acetate. Bulk concentration of the DA = 500 μM.
3.3 Experimental Procedures

3.3.1 Synthesis and Purification

*Synthesis of N-succinyldeferoxamine (N-succDFO)* The synthesis was carried out by using a modified protocol based on a previously reported work. Deferoxamine mesylate (DFO, 263 mg, 0.400 mmol, Sigma-Aldrich, St. Louis, MO) and succinic anhydride (806 mg, 8.06 mmol, Alfa Aesar, Ward hill, MA) were suspended in pyridine (4 mL) and shake overnight. This solution was then added into 40 mL of 0.015 M NaCl(aq) and mixed for 4 h. The pH of the solution was then adjusted to 2 by 12 M HCl, and then kept at 4 °C for additional 2h to facilitate the precipitating process. The solids were then collected by filtration and washed with copious 0.01 M HCl. In order to remove the remaining H₂O, EtOH was added to form azeotrope. The mixtures were concentrated *in vacuo*, and kept in desiccator overnight, yielding the white solid of N-succDFO (168 mg, 0.254 mmol, 63.5% yield). The obtained materials were stored in a −30 °C freezer.

![Scheme 3.1. Synthesis of N-succDFO](image)

*Synthesis of N-succDFO-Cys-NH₂* The peptide segment utilized were synthesized employing standard Fmoc solid-phase protocols, using Rink Amide MBHA resins (AAPPTec, Louisville, KY) at a 0.25 mmol synthesis scale. Fmoc deprotection of the Fmoc-amino acids was established by treating the resin with 4-methylpiperidine in dimethylformamide (DMF) (20% v/v). The amino acid coupling was performed after
Fmoc-deprotection, and the resin was treated with Fmoc-Cys(TrT)-OH, O-benzotriazole-
N,N,N',N'-tetramethyluronium-hexafluorophosphate (HBTU, AAPPTec, Louisville, KY),
and dissopropyl-ethylamine (DIEA) (4:4:6 mole equiv to resin) in DMF for 1 h. The
reaction completion was double-checked by a Kaiser test kit, and then the following
reaction was conducted. After another deprotection of Fmoc, the resin was mixed with
N-succDFO, PyAOP, and DIEA (2:2:3 mole equiv to resin) in DMSO to allow reaction
running overnight. The resin was then treated with a mixture of trifluoroacetic acid
(TFA), triisopropylsilane (TIS), 1,2-ethanedithiol (EDT), and H2O (92.5:2.5:2.5:2.5 v/v/v/v)
for 2 h. The liquid was then collected and added dropwise into cold ethyl ether to yield a
cloudy solution. The precipitates were then collected by filtration and washed with
copious ethyl ether. The collected solids were then kept in the desiccator overnight and
stored in a −30 °C freezer until further use without additional purification.

Scheme 3.2. Synthesis of N-succDFO-Cys-NH2

*Synthesis of 4-(pyridin-2-yldisulfanyl)butanoate* This was synthesized by a modified
protocol based on previously reported work.131 In brief, 4-bromobutyric acid (563 mg,
3.37 mmol, Fisher Scientific, Hampton, NH) and thiourea (548 mg, 7.19 mmol, Sigma-Aldrich, St. Louis, MO) were refluxed in ethanol (12.5 mL) for 4 h. After adding NaOH (1.262 g, 31.55 mmol, in 12.5 mL of EtOH) into this solution, the mixtures were refluxed for an additional 16 hr. After cooling the solution to room temperature, the solution was concentrated in vacuo, and diluted with water that was extracted twice with ethyl ether. The aqueous phase was then acidified by 4 M HCl to pH 5, resulting in a cloudy solution that was extracted by ethyl ether. The organic portion was dried with Na₂SO₄ and concentrated to yield clear, oil-like 4-sulfanylbutyric acid. 4-sulfanylbutyric acid was then dissolved in MeOH and added dropwise into a 1.5-mL methanolic solution of 2-aldrithiol (0.769 g, 3.49 mmol). The mixtures were allowed to react for 3 h and were purified by RP-HPLC. Purified products were collected and solvents removed in vacuo. The resultant yellowish oil was then dissolved in CHCl₃, dried over Na₂SO₄, and solvent removed to give HO₂C-buSS-Pyr as a light yellow oil (142 mg, 18.4% yield).

Scheme 3.3. Synthesis of HO₂C-buSS-Pyr

**Camptothecin-4-(pyridin-2-yldisulfanyl)butanoate (CPT-buSS-Pyr)** Camptothecin (CPT, 107.9 mg, 0.31 mmol, AvaChem Scientific, San Antonio, TX) and dimethylaminopyridine (DMAP, 22.4 mg, 0.184 mmol, TCI America, Portland, OR) were initially suspended in DCM (18 mL). HO₂C-buSS-Pyr (142 mg, 0.62 mmol) and diisopropylcarbodiimide (DIC, 250 μL, 1.6 mmol, TCI America, Portland, OR) were then added to the mixtures and stirred for 36 h. This solution was then filtered, diluted with chloroform (30 mL), washed
with NaHCO$_3$ (25 mL) and brine (25 mL), then dried over Na$_2$SO$_4$ and concentrated in vacuo. The crude product was purified by flash chromatography using EtOAc (250 mL) and then 0.5% MeOH in EtOAc (250 mL). Collected products were identified by TLC and underwent solvent removal in vacuo to yield a pale yellow solid (136.2 mg, 78.6% yield).

Scheme 3.4. Synthesis of CPT-buSS-Pyr

Syntheses of the DAs and Purification  The syntheses of all DAs were carried out by dissolving CPT-buSS-Pyr and N-succDFO-Cys-NH$_2$ in N$_2$-purged dimethylsulfoxide (DMSO, 1 mL) (2:1 by mol). The mixtures were allowed to react for 1 d at room temperature, and then purified by RP-HPLC. Eluted products after purification were collected, flash frozen by liquid nitrogen, and lyophilized. The purity of the collected products were confirmed by RP-HPLC and ESI-MS, and the conjugate concentrations were calibrated before aliquotting into predetermined amounts.

Scheme 3.5. Synthesis of DFO-C-CPT
3.3.2 Calibration of the Concentration

The concentration of DAs was calibrated by analyzing the reduced product from the DAs — **CPT-buSH**. A stock solution of the DA was prepared by dissolving in MeCN/H$_2$O (1:1). 5 μL of the stock DA solution was then diluted to 20 μL by MeCN/H$_2$O (1:1), and mixed with 20 μL of 1 M 3,3′,3′′-phosphanetriyltripropanoic acid (TCEP, VWR, Bridgeport, NJ) for 45 min. The resultant solution was then analyzed by RP-HPLC, monitoring the absorbance at 370 nm. The CPT concentration of the analyte solution was then calculated from the area under the curve in the chromatogram by comparing with the standard solutions based on the content of **CPT-buSH**. The conjugate concentration of the stock solution was then determined based on the applied dilution and the number of CPT molecules.

3.3.3 Transmission Electron Microscopy (TEM)

The TEM samples were prepared by negative staining techniques. Before characterizations, solutions were heated in 80 °C sand bath for 10 min. The solutions (7 μL) were initially loaded on copper grids covered with carbon film. The liquids were then removed by filter papers, and 2% uranyl acetate solutions (7 μL) were subsequently deposited on the samples. The uranyl solutions were then removed by filter paper s after 30 s. The specimens were air-dried prior to imaging. Bright-field TEM imaging was performed on an FEI Tecnai 12 TWIN electron microscope (FEI, Hillsboro, OR) at 100 kV. The micrographs were acquired by a SIS Megaview III wide angle camera.
3.3.4 Cryogenic-Transmission Electron Microscopy (Cryo-TEM)

Specimens of cryo-TEM imaging were prepared by Vitrobot (FEI, Hillsboro, OR). The solutions were initially loaded on a copper grid with Lacey carbon film (Electron Microscopy Sciences, Hatfield, PA) in the controlled humidity chamber, and were blotted by the filter papers that were mounted on the Vitrobot from the both sides of the grid. This process engenders a thin film of solutions that adhere on the sample grid. The blotted samples were then transferred into liquid ethane, and were stored in liquid nitrogen until further use. Sample imaging was conducted on a FEI Tecnai 12 TWIN electron microscope (FEI, Hillsboro, OR) at 100 kV. The micrographs were acquired by a 16 bit 2K×2K FEI Eagle bottom mount camera.

3.3.5 Circular Dichroism Spectroscopy

The CD spectra were monitored by using a Jasco J-710 spectropolarimeter (JASCO, Easton, MD). The measurements were conducted immediately after diluting the DA solution into 25 μM from a freshly prepared 500-μM stock solution that was heated in 80 °C sand bath for 10 min. Solutions were loaded in a 2-cm cuvette and the spectra were recorded in the far-UV and Vis region.

3.3.6 UV-Vis Spectroscopy

The UV-Vis spectra were monitored by using an Agilent Cary 300 Bio UV-Vis spectrometer (Agilent, Santa Clara, CA) in the UV-Vis region. The solutions were loaded in a 1-cm cuvette. Displayed spectra were substrate by the background signals.
3.3.7 Degradation Studies

The degradation of DFO-C-CPT in the absence/presence of GSH was evaluated using RP-HPLC. In brief, 100 μM of DFO-C-CPT solutions were freshly prepared by using sodium phosphate buffer (pH 7.4, 20 mM) with or without 1 equiv (mole relative to the drug-amphiphile) of FeCl₃. A glutathione (GSH) stock solution was initially prepared at 20 mM in sodium phosphate buffer, and the pH was adjusted to 7.4 after dissolving the GSH. This solution was then added into the DFO-C-CPT solutions with equal volume, and incubated at 37 °C water bath (final concentration: 50 μM DFO-C-CPT, 10 mM GSH). Solutions were sampled after 0.5, 1, 2, 4, 6, 8, and 24 h of incubation. The samples were acidified by the addition of 0.4 μL of 12M HCl, flash frozen with liquid N₂ and stored at −30 °C until further analysis. The amounts of remained DAs were quantified by determining the area under the curve in the chromatogram (monitored wavelength: 362 nm) through an RP-HPLC system equipped with a Varian Pursuit XRs C18 column (5 μm, 150 × 4.6 mm). Data were then plotted as a percentage of the original concentration.

3.3.8 Cytotoxicity Studies of the Drug-Amphiphiles

The cytotoxicity of all synthesized conjugates was evaluated on a human breast cancer cell line (MCF-7), and a human prostate cancer cell line (PC3 flu). In brief, cells were initially seeded into 96-well plates at 5000 cells/well and incubated in the medium – DMEM containing 10% FBS and 1% of an antibiotics solution (penicillin and streptomycin) – for 12 hours at 37°C in a 5% CO₂ atmosphere (Oasis CO₂ incubator, Caron, Marietta, OH). The medium was then replaced with freshly prepared medium.
containing varying concentrations of conjugates using free camptothecin as control, and incubated for a further 48 hours. Cell viability was determined using the SRB assay (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions. The IC50 (the half maximal inhibitory concentration) values for the DA in each condition were obtained by fitting the data using the sigmoid or Hill equation curve analysis functions within IGOR Pro (Wavemetrics Inc., Lake Oswego, OR).

3.4 Results and Discussions

This molecule, DFO-C-CPT, bears overall amphiphilicity since the CPT is relatively more hydrophobic. It was observed that DFO-C-CPT self-assembles into filaments when dissolved in mini-Q water (Figure 3-1b), while this DA assembled into nanotubes if 1 equiv (mole relative to the DA) of Fe(III) was present in aqueous media (Figure 3-1c–d), as visualized by transmission electron microscopy (TEM) and cryogenic-TEM (cryo-TEM). The outer diameter of these nanotubes was 144 ± 20 nm as measured from cryo-TEM micrographs. Notably, the transformation of filaments to tubes of supramolecular morphology can be accomplished by adding Fe(III) into a DFO-C-CPT solution that possessed nanofilaments (Figure 3-1e). Incubation of DFO-C-CPT with insufficient Fe(III) would lead to coexistence of nanofilaments and nanotubes (Figure S3-5 in SI). The thickness of the filaments (measured from the narrowest distance in the turnover position in TEM micrographs) is 10.7 ± 2.2 nm. The measured wall thickness for the self-assembled nanotubes can be up to 20 nm, a dimension greater if the wall is the bilayered
structure when the DFO-C-CPT is fully extended and implying these nanotubes possess multiple stacked layers.

UV-Vis and Circular dichorism (CD) spectrometric studies provide insight to the molecular level packing and the chirality of the system. Remarkably, DFO-C-CPT exhibits different UV-Vis and CD patterns in its spectra when Fe(III) is presence (Figure 3-2). Since the signals are from the CPT moiety, the observed discrepancy is believed to be the change of CPT interactions in self-assembled states that are triggered by Fe(III) chelation. The observed red shift in UV-Vis spectra of DFO-C-CPT solutions in which nanotubes were formed in the presence of Fe(III) may be indicative of the DAs arranging with more regular supramolecular packing, which leads to larger $\pi$ electron delocalization and reduced energy gap between highest occupied molecular orbits (HOMO) and lowest unoccupied molecular orbits (LUMO). The higher absorbance of DFO-C-CPT in H$_2$O at monitored range is due to the non-specified scattering that reduces transmittance of the solution, since the solution was still turbid upon the instant dilution from a concentrated stock solution.

The CD spectrum of DFO-C-CPT in H$_2$O displayed different characteristics especially in regions above 350 nm, a regions more related to the $\pi$$-$$\pi^*$ transition of the chromophore CPT. The constant negative signal above 410 nm of DFO-C-CPT in H$_2$O is believed be the random diffractions from CPT aggregation, since this constant signal vanish rapidly in more diluted conditions without additional peak (see S4 in SI). On the other hand, if Fe(III) was chelated that triggered the formation of tubes, there was an additional, broad, negative peak at ~405 nm in CD spectrum. This pattern is assumed to
be associated with the intermolecular interactions between the CPT moieties when CPT conjugates assembled into tubular structures, as shown in our previous work. Other differences in CD spectra included the bisignate signals at 250–260 nm and 350–380 nm while the nanotubes were formed, which induced by the intermolecular chiral packing, in our case, from the CPT. On the other hand, no obvious exciton coupling was detected in DFO-C-CPT in H₂O. In addition, it was also noted that there is no direct observation of signatures of β-strands whether the materials is dissolved either in H₂O or 1 equiv Fe(III), although there is still a cysteine residue in our molecular design that could potentially lead to the formation of hydrogen bonding. Our results in CD spectra suggest the observed one-dimensionality of assembled structure may be dominantly driven by the intermolecular π-π interactions among the monomers.

**Figure 3-2. Spectroscopy studies of DFO-C-CPT in 1 equiv of Fe(III) or H₂O.** CD (top) and UV-Vis (bottom) spectra of DFO-C-CPT. Samples were monitored at the stock concentration of 25 μM instantly diluted from a 500 μM stock solution that were freshly prepared. The UV-Vis data presented were subtracted by the background from a 25 μM Fe(III) aqueous solution and H₂O respectively.
Intermediate structures toward the development of tubular structure often provide insights to the mechanism of self-assembling processes. Occasionally, in the presence of Fe(III), a few ribbon-wrapped structures can be observed (Figure 3-3a). Helical ribbons are often regarded as the precursors of the tubular structures in self-assembly systems through width-widening or pitch-shrinking processes. It was also noted the pitch of different helical ribbons formed by DFO-C-CPT was fixed given the width varied, implying the formation of tubes from helical ribbons is the result of width-widening of ribbons (Figure 3-3b and Figure S3-6 in SI).

In addition, it could be clearly seen the thickness variation of ribbon-wrapped tubes (Figure 3-3a), serving as a direct evidence that the most observed nanotubes comprise multi-walled construct, in accordance with our previous dimension estimations. The multiwalled construct of nanotubes could also be seen in cryo-TEM where some of the thicknesses of the nanotubes were larger than those of some other spotted tubes (Figure 3-3c). The mechanism of formation of multi-walled architecture may be very similar to the multilaminar layers observed in concentrated surfactants. Interestingly, these two major characteristics are found to resemble to the multi-walled nanotubular construct formed by the catanionic mixtures of CPT-conjugated DAs, where the surface curvature of assembled 1D structures reduces as ion-paired possess different packing geometry. We believe the morphological transformation from filaments to tubular structures upon chelation can be explained by the change of packing geometry as well. The presence of Fe(III) is assumed to cause intermolecular interactions with the hydroxamate groups on the DFO domain, where the coordination of Fe(III) lead to the change of overall
packing geometry from a more conical like geometry into a less conical like one (Figure 3-3d). In brief, the formation of these multi-walled nanotubes is the cumulative effects of two phenomena: 1) widening of helical ribbons and 2) formation of the multilayers, given the one-dimensionality is maintained.

Figure 3-3. Proposed mechanism of morphological change triggered by Fe(III) coordination according to electron microscopy. a) A helical-ribbon-wrapped nanotube that explicitly shows the thickness variations in TEM imaging. White arrows indicate the scales of winding helical ribbon. b) TEM micrograph of a ribbon-wrapped nanotube with attenuating width of the ribbon. The width, $w$, of the ribbons decreases near the ribbon end while the pitch, $p$, remains constant. c) Schematic representation of the evolution of a helical ribbon into tubes along with the width-widening processes after the Fe(III) chelation (c) The cryo-TEM micrograph of direct observation of a multiwalled nanotube formed by DFO-C-CPT (indicated with white arrow). (d) Proposed mechanism coordination triggered change in packing geometry, and the formation of multiwalled nanotubes as a result of width-widening of ribbons and multilayer stacking once the one-dimensionality is maintained. Bars = 200 nm.
This designed molecule, as a therapeutic DA, also possess effective cytotoxic efficacy toward cancer cells \textit{in vitro} with or without the chelation of Fe(III) (evaluated by the survival percentage while incubating the DA with metastatic pancreatic cancer cell line PC3FLU, and breast cancer cell line MCF-7). Upon chelation, there is no significant reduction of cytotoxicity of the chemotherapeutics compared with the cytotoxic effects of DA in H\textsubscript{2}O (Figure 3-4). Our results also suggest there is no distinct difference in terms of cytotoxicity effects whether the experiments were conducted by dilution into DMEM of DA that was initially dissolved in dimethylsulfoxide or directly reconstituted by DMEM as well. Degradation study of the DA shows that pure CPT is available by incubating \textbf{DFO-C-CPT} with glutathione (GSH), which is involved with the direct breakdown of disulfide bonds within the DA, and the cyclization-assisted hydrolysis of ester bond in the \textsf{buSS} linker at physiological pH (See Figure S3-5 in SI).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3-4.png}
\caption{Cytotoxicity evaluations of DFO-C-CPT. Survival rates for the breast cancer cell line, MCF-7 (a), and the pancreatic cancer cell line, PC3 flu (b).}
\end{figure}
3.5 Conclusions

In this study, we have successfully designed a $\pi$-domain-rich molecule with a siderophore-derived domain that is capable of undergoing filament-to-tube transition of the assembled-structure upon chelation. We believe this design strategy can be applied in constructing smart materials, or can be regarded as an effective strategy to fabricate self-assembled nanotubes in bottom-up systems in multi-disciplinary applications.

3.6 Acknowledgements

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Designing Supramolecular Polymers through a Miktoarm Star Construct of Small Molecules†

4.1 Abstract

We report here the design and synthesis of an ABC miktoarm star peptide connecting through a lysine junction a short peptide sequence and two hydrophobic but immiscible blocks (a hydrocarbon and a fluorocarbon). The designed molecule can self-assemble into one-dimensional nanostructures with a great diversity of kinetically evolving morphologies in aqueous solution, while molecules that contain only one of the two hydrophobic blocks form structurally similar filaments. We believe the rich assembly behavior and morphological evolution are a direct reflection of the molecular frustration present within the filament core as a result of the incompatibility of the fluorocarbon and hydrocarbon segments. Our finding opens new opportunities for creating complex supramolecular polymers through the architecture design of small molecular building units.

4.2 Introduction

The spontaneous association of small molecules into discrete one-dimensional nanostructures with a high degree of internal order provides a facile yet effective means to construct supramolecular polymers.5, 23, 24 Such supramolecular polymers are

† Reproduced with permission from Lin, Y.-A.; Ou, Y.-C.; Cheetham, A. G.; Cui, H. Supramolecular Polymers Formed by ABC Miktoarm Star Peptides, ACS Macro Lett., 2013, 2, 1088–1094. (DOI: 10.1021/mz400535g). Copyright 2013 American Chemical Society.
characterized with anisotropic growth governed by directional, non-covalent interactions, possessing interesting optical, electrical or biological properties. This bottom-up strategy has led to the creation of a plethora of functional nanomaterials, including bioactive nanofibers formed by peptide amphiphiles, and semiconducting or photoconductive nanowires and nanotubes formed by \( \pi \)-conjugated molecules. Since the macroscopic/nanoscopic properties of the resulting materials are strongly associated with the chemical structures and organization of the building blocks, bridging the design rationale of building blocks and the supramolecular characteristics becomes crucial for the development of functional nanomaterials. Peptides are regarded as very effective molecular building blocks, serving as powerful bottom-up constituents to construct supramolecular polymers. Accordingly, much effort has been devoted to elucidating connections between peptide sequences and resulting supramolecular morphology. However, design parameters for the hydrophobic segments, such as the chain architecture (branched vs. linear), though being an effective strategy frequently used in polymeric systems to achieve novel morphologies, are rarely explored for small molecular self-assembly. In particular, incorporation of a third immiscible component has not been exploited specifically in the design of peptidic building units. In this work, we report the first example of an ABC miktoarm star peptide that can self-assemble into a variety of complex supramolecular polymers in aqueous conditions.

For small molecules to assemble into stable, discrete one-dimensional nanostructures that are not necessarily stabilized by a spontaneous curvature, we
believe at least two criteria should be included in the molecular design. One part of the molecule should afford a strong and directional associative interaction (e.g. hydrogen bonding\textsuperscript{21, 129, 140} or \(\pi-\pi\) interactions\textsuperscript{130, 134-136, 147, 148}) for one-dimensional growth into stable assemblies. A second feature should also be included to limit growth in other dimensions(s) so as to form discrete assemblies. Inspired by the miktoarm star terpolymer system developed by Lodge, Hillmyer and coworkers,\textsuperscript{144-146} we incorporated the design principles to create supramolecular polymers formed by an ABC miktoarm star peptide, an amphiphilic molecule comprising three distinct segments linked through a lysine junction: 1) a saturated hydrocarbon, 2) a hydrophobic and lipophobic fluorocarbon, and 3) a short peptide sequence (Figure 4-1a). The use of two different hydrophobic blocks, immiscible with one another, could potentially offer a new mechanism to tune the morphology when the hydrophobic collapse forces these two moieties into close proximity. The peptide GNNQQNY was chosen because of its high propensity to form parallel \(\beta\)-sheets;\textsuperscript{103} this seven-residue peptide is derived from the key amyloid-forming region in yeast prion Sup35, known to adopt a parallel \(\beta\)-sheet secondary structure in the native protein.\textsuperscript{103} In addition, two glutamic acid residues were placed at the C-terminus of the peptide to impart hydrophilicity to the designed peptides.
Figure 4-1. Chemical structures and schematic representations of the studied miktoarm star peptide FHP and the two control peptides, FP and HP, that possess only one of the two hydrophobic blocks used in this study.

4.3 Experimental Procedures

4.3.1 Peptide Synthesis & Purification

All peptides used in this work were synthesized by utilizing standard Fmoc solid-phase peptide chemistry on Fmoc-Glu(OtBu)-Wang resin (AAPPTec, Louisville, KY) with an AAPPTec Focus peptide synthesizer. In brief, in each amino acid coupling cycle, the resin first underwent a Fmoc-deprotection by treated with 20% 4-methylpiperidine (Alfa Aesar, Ward Hill, MA) in DMF, and was sequentially treated with activated Fmoc amino acid by using 4 Equiv of O-(Benzotriazol-1-yl)-N,N,N’,N’- tetramethyluronium
hexafluorophosphate (HBTU) (AAPPTec, Louisville, KY), 6 Equiv of diisopropylethylamine (DIEA) (Alfa Aesar, Ward Hill, MA). The miktoarm star construct of FHP was synthesized by using the following orthogonal chemistry: Fmoc-Lys(Mtt)-OH was coupled at the N-terminus where the Fmoc and Mtt group could be deprotected independently. The Mtt-protected ε-amine group can be deprotected by using TFA/TIS/DCM (3:5:92 by vol), while the Fmoc group can be removed by using 4-methylpiperidine/DMF (1:4 by vol). Functionalization with the fluorocarbon or hydrocarbon was performed on-resin after deprotecting respective amino group. The deprotected resin was allowed reacting with 4 Equiv of 4,4,5,5,6,6,7,7,8,8,9,9,9-tridecafluorononanoic acid (Sigma-Aldrich, St Louis, CO) or 4 Equiv octanoic acid (Sigma-Aldrich, St Louis, CO) that were respectively activated by 4 Equiv of HBTU and 6 Equiv of DIEA in DMF. Regarding to the synthesis of FP and HP, acetylation was respectively performed for the ε- or α-amine group by treating the resin with acetic anhydride/DMF (1:4 by vol). Cleavage of the peptides from the resin was accomplished by treating the resin with TFA/TIS/H₂O (95:2.5:2.5 by vol) for 3 hours. The cleavage solution was then collected and concentrated in vacuo. This concentrated solution was then added dropwise into cold ethyl ether to precipitate the crude materials. Purification of the crude materials was performed on a preparative HPLC column by using a gradient of H₂O/MeCN both containing 0.1% of ammonium hydroxide at pH 9. The purified fractions were collected and analyzed by matrix assisted laser desorption-ionization (MALDI-ToF) mass spectrometry. Collected liquids were then lyophilized to
yield the dry mass. All lyophilized peptides were stored in a −20 °C freezer until further use.

4.3.2 Circular Dichroism

The CD spectra for all peptides were monitored by JASCO J715 spectropolarimeter in the far UV region (190–260 nm). Each solution was loaded in 0.1 mm detachable cuvette. The mean residue ellipticity \([\theta]\) was calculated by the following equation

\[\theta = \frac{\theta}{\ell C n}\]

where \(\theta\) is the obtained ellipticity in deg, \(C\) is the concentration of the peptide calibrated by the absorbance at 275 nm originated from the tyrosine residue (extinction coefficient = 1390 AU cm\(^{-1}\) mmol\(^{-1}\) mL\(^{-1}\)), \(\ell\) is the light path length of the cuvette, and \(n\) is the number of amino acid residues.

4.3.3 Determination of Critical Micellization Concentration (CMC)

The determination of CMC values was conducted based on the modified protocol of a reported work.\(^{149}\) The stock solution of Nile red was initially prepared by dissolving the dye in acetone. Identical volume of the Nile red solution was loaded in centrifuge tubes, where the solvent evaporates under room temperature to yield the dry mass of Nile Red. Various concentrations of the peptide solutions were prepared in H\(_2\)O, and then added into the centrifuge tubes with equal amount of Nile red solids (the final bulk concentration of Nile red was made up to 1 \(\mu M\)). These mixtures of Nile red and peptides were then vortexed, and incubated in the dark at room temperature overnight to assume equilibrium. Fluorescent spectra of Nile red were then monitored by a
Fluorolog fluorometer (Jobin Yvon, Edison, NJ) with fixed excitation wavelength at 560 nm; spectra were monitored within 580–720 nm. The ratios of the emission intensity at 635 nm (near the emission maximum when Nile Red was encapsulated in the hydrophobic environments) to that at 660 nm (the weak emission maximum in aqueous conditions) were then plotted against the peptide concentrations. Since the Nile Red was strongly quenched in aqueous conditions, the surge in intensity ratio (I_{635}/I_{660}) indicates the dye being encapsulated in the hydrophobic environments in assembled structures, whereas this transition occurs as the incubated peptides exceed their CMC values. The CMC values were determined by calculating the crossover point of the ratio curve plotted against the peptide concentrations before/after the intensity surge.

4.3.4 Transmission Electron Microscopy (TEM)

The TEM specimens were prepared by initially loading 5 μL of peptide solutions on copper grids with supporting carbon film (Electron Microscopy Sciences, Hatfield, PA), and excess liquids on the grid were subsequently removed by filter papers. The specimens were then loaded with another 5 μL of 3% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA), and were blotted by filter papers to remove the excess liquid. After the specimens were air-dried, TEM imaging were then carried out on a FEI Tecnai 12 TWIN transmission electron microscope equipped with SIS Megaview III wide angle camera at 100kV.

4.3.5 Cryogenic Transmission Electron Microscopy (cryo-TEM)

Specimens used for cryo-TEM imaging were prepared by FEI Vitrobot system within the humidified chamber. Initially, 6 μL of the peptide solutions were loaded on a
Copper grid with Lacey Carbon Film (Electron Microscopy Sciences, Hatfield, PA). After the solution formed a layer of water film on the copper grid, the specimens were blotted from both sides with filter papers, and transferred into liquid ethane that was cooled by liquid nitrogen. The frozen samples were then transferred and stored in liquid nitrogen before usage for imaging. The imaging facilities were identical to those used in TEM imaging depict above, and the micrographs were acquired by a 16 bit 2K×2K FEI Eagle bottom mount camera.

4.3.6 Atomic Force Microscopy

Specimen used for AFM topological analysis was prepared by depositing 2 μL of peptide solution on freshly peeled surface of mica disk adhered on a specimen disk (Ted Pella, Inc., 12 mm). Topological analysis was performed after the specimens were air-dried by using a Brucker Dimension Icon atomic force microscope under ScanAsyst automatic tapping mode. The automatic tapping mode was carried out in air with high-resolution probes (SNL-10, C triangular, f: 40-75 kHz, k: 0.58 N/m, Veeco, NY). Height analysis was conducted by using Nanoscope-SanAsys software.

4.4 Results and Discussions

The miktoarm star peptide, FHP, was synthesized using the standard protocols of Fmoc solid-phase peptide synthesis. Two control molecules (FP and HP), containing only one of the two hydrophobic moieties, were also synthesized in order to better understand the assembly behaviors of the miktoarm star peptide (Figure 4-1b–c). We first determined the critical micellization concentrations (CMC) of the three molecules
using Nile red as a probe. Nile red is a lipophilic, solvatochromic dye that fluoresces intensely upon exposure to hydrophobic environments, whilst in aqueous media its fluorescence is strongly quenched and red shifted.\textsuperscript{149, 150} The CMC of the studied peptides was determined by incubating these molecules at various concentrations with a fixed content of Nile red. Plotting the ratio of intensity at 635 nm (emission maximum of the encapsulated dye) to that at 660 nm (emission maximum in aqueous conditions) against the concentration of each peptide shows the transition that occurred when the peptide concentration exceeded the CMC (Figure 4-2a–c; complete spectra are given in Figure S4-4 in SI). These experiments show that FHP, as expected due to its greater hydrophobicity (two tails versus one), has a lower CMC value (~1 μM) than that of both FP and HP (~10 μM for both). There was no apparent difference in CMC value between FP and HP. At concentrations above their respective CMC values, all three peptide-conjugates assume a β-sheet conformation in aqueous solution, as revealed by our circular dichroism (CD) spectrometric studies (Figure 4-2d). All three spectra are characterized by a negative peak in the 215–220 nm region and a positive peak around 195 nm, both signatures of the β-sheet secondary conformation.
Figure 4-2. Characterizations of designed peptides. Determination of CMC for FHP (a), FP (b) and HP (c) using the Nile red encapsulation method. (d) Far-UV circular dichroism spectra of 0.1% (w/v) solutions of FHP, FP, and HP, respectively, all showing characteristic absorption of β-sheet assemblies.

Characterization of the assembled nanostructures in aqueous solutions was accomplished using both transmission electron microscopy (TEM) and cryogenic-TEM (cryo-TEM). Regular TEM imaging of negatively stained samples offers a relatively higher resolution, while cryo-TEM enables direct imaging of nanostructures embedded in a thin layer of vitrified sample. Figure 4-3 depicts TEM and cryo-TEM micrographs of nanostructures formed by FHP, FP and HP after one day of incubation. The one-dimensionality of the observed nanostructures was interpreted to result from the intermolecular hydrogen bonding among the peptide units, in accordance with our CD measurements.
Two major types of filaments were identified for FHP assemblies in both TEM and cryo-TEM imaging—twisted ribbons and helical ribbons—after one day of aging, with twisted ribbons observed much more frequently (Figure 4-3a–d). Supramolecular structures of FHP identified as twisted ribbons were those comprising a periodic pitch and twisting with a rotation direction perpendicular to the long axis of the ribbon (Figure 4-3c). We noted that the thickness of these twisted ribbons (measured from
regular TEM micrographs) was on average ~15 nm, a value that is larger than the expected thickness of a partially or fully interdigitated bilayer, implying the observed ribbons may consist of multiple stacked layers. The pitch of the twisted ribbons measured from TEM micrographs was ~90 nm. Helical ribbons of FHP could be distinguished from the twisted ribbons as their distinct two-dimensional projections were clearly visible in both TEM and cryo-TEM imaging (Figure 4-3a-b and 4-3d). The observed narrow helical ribbons have a pitch on the order of hundreds of nanometers and a ribbon width of ~30 nm.

Both FP and HP were also found to form filamentous structures in aqueous solutions. For FP, both twisted ribbons (twisting with periodic pitch) and single filaments (no obvious pitch) were observed to be the dominant nanostructures. The twisted ribbons formed by FP possess thicknesses that are almost identical to the size of the single filaments (7.0 ± 1.4 nm), suggesting the ribbons could be the intertwined form of the single filaments. HP assembled into fibers with a width of 13.6 ± 2.5 nm, a dimension close to the doubled value of the estimated length of the fully extended peptides (assuming a β-sheet conformation), inferring that HP assembles in a core-shell manner. This molecular arrangement is in consistency with the proposed core-shell cylindrical structure formed by the majority of reported peptide amphiphiles.\(^{17, 18, 138}\)
Figure 4-4. Kinetic evolution of the supramolecular polymers formed by FHP after aging the samples for 6 months. Representative cryo-TEM (a) and TEM (b) micrographs collected from a 0.1% FHP aqueous solution aged for 6 months. Arrows in panel b indicate the twisted-to-helical transitions of intermediate structures. (c) Scatter charts of the correlation between ribbon pitch and width (left), and the correlation between ribbon pitch and thickness (right) measured for twisted ribbons from a 0.1% FHP aqueous solution that was aged for 1 day or 6 months. (d) Correlation between the pitch and width for twisted ribbons, type I helical ribbons and type II helical ribbons formed by FHP after 6 month aging. \( n = 100 \) for twisted ribbons and \( n = 30 \) for type I and type II helical ribbons. Representative TEM images of helical ribbons (e, f) and an intermediate twisted-to-helical ribbon (g) after 6 month aging, where the black arrow indicates the transition spot. All bars = 100 nm.

For self-assembling systems in which building blocks possess strong intermolecular interactions, the observed morphologies are often only kinetically stable at the
experimental timescale and could potentially evolve into thermodynamically more stable structures in later stages. Short- or long-term aging, for instance, can lead to the transformation of twisted ribbons into helical coils or nanotubes.\textsuperscript{151-154} We therefore investigated the kinetic evolution of the assemblies formed by the miktoarm star peptide FHP, and observed significant changes in the supramolecular structures after aging the solution for 6 months. First of all, a general increase in width was evident for twisted ribbons from both TEM and cryo-TEM imaging upon aging (Figure 4-4a–b and Figure S4-5 in SI). Second, the pitch and thickness of the twisted ribbons were also found to increase over time (Figures 4-4c-d). The thickness of the ribbons formed by FHP could be up to \( \sim30 \) nm (averaged thickness \( \sim20 \) nm), implying time-dependent molecular stacking in this dimension. We also found that there is an approximate linear correlation between thickness and pitch and between width and pitch for these twisted ribbons, suggesting that the evolution of twisted ribbons observed here may be similar to the process of hierarchical assemblies formed by chiral rod-like molecules rich in \( \beta \)-strands.\textsuperscript{155} Third, helical ribbons were much more frequently observed after aging, accompanied with an increase in dimensions (Figures 4-4e-f and 4-5).

Closer examination further reveals that the helical ribbons generally possess widths above 30 nm, while the twisted ribbons usually comprise widths less than this value (Figure 4-4c). In addition, several TEM images seem to capture a number of intermediate structures that were in the process of converting from twisted ribbons to helical ribbons (Figure 4-4g, and S4-5b), and these intermediate structures seldom possess a width greater than 30 nm. These observations indicate that there might exist a critical value in
ribbon width above which twisted ribbons would commence to transform into helical ribbons. The twisted ribbon-to-helical transformation has been reported possible through theoretical simulation,\textsuperscript{156} and also in experimental work where the morphological transition was ascribed to the chiral instability of twisted ribbons during the widening process.\textsuperscript{151-154} These results led us to propose the possible mechanism shown in Figure 5b, the illustration depicting the transformation from twisted ribbons to helical ribbons. Despite precedent studies indicating the possibility for helical ribbons to transform into tubules during the width-widening process,\textsuperscript{152,157} there were no indication for forming tubular structures in our system.

Interestingly, a third type of large one-dimensional structures was also frequently spotted for FHP samples aged for 6 months, referred here as “type II helical ribbons”, with alternating contrast along the long axis (Figure 4-5c–e, topological analysis in Figure 4-5f and Figure S4-6). The type II helical ribbons generally possess a width greater than 30 nm and in several cases up to ~100 nm (Figure 4-5a). The fraction of helical ribbon II out of the total nanostructures observed was approximately 51.8% on the basis of our statistics from 10 TEM images (total count: 251). It is important to note that all the samples prepared for regular TEM imaging in this study were stained with a 3% (w/v) uranyl acetate aqueous solution and, as a consequence, the contrast mechanism of the collected TEM micrographs is primarily dominated by the staining reagent, with the brightest areas generally having the least deposition of the uranyl ions. Unlike helical ribbons discussed previously (type I) that demonstrate bright spots symmetrically located on both sides (Figure 4-4e–f), the type II helical ribbons reveal
only alternating bright spots and gray regions along the ribbon’s long axis (Figure 4-5a–c).

However, there are several common features between type II and type I helical ribbons: first, the type II helical ribbons possess a similar width-and-pitch relation to that of type I helical ribbons (Figure 4-4c). Second, intermediates structures were also spotted revealing morphological transition from twisted ribbons to type II helical ribbons within the same filamentous nanostructures (Figure 4-5g). In addition, both type I and type II helical ribbons exhibit the right-handed supramolecular chirality, evidenced by both TEM and AFM imaging (Figure S4-6). It should be noted that the supramolecular chirality could only be determined from TEM micrographs if the orientations of the grids of the negatively stained specimens were known with respect to the electron beam travelling direction. These observations suggest that type II and type I helical ribbons are likely the same structures in origin. The distinct contrast between the two structures observed in TEM images (collected from samples stained with uranyl ions) could originate from the difference in the deposition of the staining agents. We speculate that type II helical ribbons are likely the later stage assemblies of the type I helical ribbons with both increased thickness and widths. Their more robust structures due to the increased thickness would prevent the collapse of the ribbon structure during the sample preparation while the increased width likely prevents the deposition of the uranyl ions into the tubular inner channels, leading to formation of different contrast patterns in TEM images. Occasionally, we could observe two or more of these structures
interact or intertwine with each other to form more complex, higher hierarchical assemblies (Figure 4-5h–i).

Figure 4-5. Proposed kinetic evolution and spotted type II helical ribbons formed by FHP. a) Statistical width distribution of twisted ribbons, helical ribbon I and helical ribbon II after aging the solution for 6 months (n=50 for each type of ribbon). A Gaussian distribution fits the data (black curve). b) The proposed transition mechanisms from twisted ribbons to helical ribbons and the possible pathways for the observed pitch and width variations associated with sample aging (top), and also a proposed model for the chain packing within the observed FHP helical ribbons (bottom). (c–e) Representative TEM micrographs of the twisting stacked ribbons formed by FHP after aging 6 months, displaying the right-handedness according to the uranyl accumulation on the surface. (f) Topological analysis in TEM for a twisting stacked ribbon formed by FHP. Top panel depicts the instrumentation setup for the topological studies. Bottom panels display the projection of a twisting stacked ribbon while the specimen is tilted in TEM column. Tilt angle $\alpha$ is $-45^\circ$, $0^\circ$, and $45^\circ$ for the left, middle and right panels. (g–i) TEM micrographs of an intermediate structure bearing twisted ribbon and type II helical ribbon (panel g, the arrow indicates the transition), and intertwining one-dimensional structures (h,i). All bars =100 nm.
In contrast to the rich assembly behaviors of the FHP peptide, the two control molecules, FP and HP, did not exhibit any obvious morphological changes after 6 months of aging (Figure S4-7 in SI). Replacing the fluorocarbon segment of FHP with a hydrocarbon gave an amphiphile (H₂P) that was observed to assemble into nanofibers similar to those formed by HP, and that did not show any kinetically dependent assembly behavior (see Figure S4-9 in SI).

These results clearly suggest that the difference in self-assembly among FHP, FP, and HP, and in particular the observation of a variety of different supramolecular polymers formed by FHP, is directly linked to the immiscibility between the hydrocarbon and fluorocarbon segments within the FHP assemblies. Though this immiscibility has been reported to lead the ABC miktoarm star terpolymers to form chemically distinct multi-compartments within polymeric micelles, there was no direct observation of multiple compartments within the formed one-dimensional nanostructures in our case. Given the extremely short lengths of the used fluorocarbon and hydrocarbon, it might be impossible to access the separate domains comprising only hydrocarbons or fluorocarbons (Figure 4-5b). However, we did observe, particularly in the sample aged for six months, that the width of the FHP assemblies could vary dramatically within a single filament (Figures 4-4 and 4-5). Amyloid peptides are well known to form polymorphic filaments of varying twists and widths; however, their width is often consistent across a large length scale within a particular filament. The heavily fluctuating width observed within a single filament in our system manifest again the effect of having two immiscible hydrophobic chains in the molecular design.
Although the studied system is concerned with small molecular building units, it bears some resemblance to a polymeric system. It has been shown by several laboratories that small molecules could demonstrate kinetically controlled aggregation behaviors similar to the macromolecular assemblies.\textsuperscript{151-153, 158-160} Our system appears to be another example where kinetic evolution plays a critical role in the creation of supramolecular polymers. More importantly, our results suggest subtle change in the molecular design could lead to a profound effect on the assembled nanostructures. In polymeric assembly systems, the segregation force between different polymer chains is determined by the dimensionless product $\chi N$, where $\chi$ is the Flory-Huggins interaction parameter describing the interactions between two different monomers and $N$ is the degree of polymerization. In our oligomer system, while $\chi$ could be relatively large due to the strong incompatibility between fluorocarbons and hydrocarbons\textsuperscript{161}, $N$ is an extremely small number (only involving 6 fluorocarbons in our system, $N=3$). Therefore, the product $\chi N$ would be too small to lead to any significant chain segregation, and thus may not be used to explain exclusively the observed morphological difference. However, the observation that this subtle immiscibility can induce a dramatic change in the assembly landscape to produce a wide variety of interesting nanostructures is, therefore, truly remarkable and points to the vast potential of the molecular design in low-molecular-weight building units for the development of supramolecular materials.
4.5 Conclusions

In summary, we have presented a design of branched peptide that possesses both fluorocarbon and hydrocarbon segments. These immiscible chains lead to the spontaneous formation of complex supramolecular polymers that exhibit a time-dependent evolution from twisted ribbons to helical ribbons. The design of architecture for small molecular building units could provide an effective approach to construct alternative morphologies with similar surface chemistry through the incorporation of different hydrophobic or lipophoboc segments.

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5 Design and Incorporation of Matrix-Metalloproteinase Degradable Features into Supramolecular Filament Networks‡

5.1 Abstract

One-dimensional nanostructures formed by self-assembly of small molecule peptides have been extensively explored for use as biomaterials in various biomedical contexts. However, unlike individual peptides that can be designed to be specifically degradable by enzymes/proteases of interest, their self-assembled nanostructures, particularly those rich in $\beta$-sheets, are generally resistant to enzymatic degradation because the specific cleavage sites are often embedded inside the nanostructures. We report here on the rational design of $\beta$-sheet rich supramolecular filaments that can specifically dissociate into less stable micellar assemblies and monomers upon treatment with matrix metalloproteases-2 (MMP-2). Through linkage of an oligoproline segment to an amyloid-derived peptide sequence, we first synthesized an amphiphilic peptide that can undergo a rapid morphological transition in response to pH variations. We then used MMP-2 specific peptide substrates as multivalent cross-links to covalently fix the amyloid-like filaments in the self-assembled state at pH 4.5. Our results show that the cross-linked filaments are stable at pH 7.5 but gradually break down into much shorter filaments upon cleavage of the peptidic cross-linkers by MMP-2. We believe that the

reported work presents a new design platform for the creation of amyloid-like supramolecular filaments responsive to enzymatic degradation.

5.2 Introduction

Peptides with β-sheet forming sequences derived from, or inspired by, amyloid proteins, have been extensively incorporated into various molecular building units to construct self-assembling one-dimensional (1D) nanostructures.17, 20, 21, 139, 162 These 1D amyloid-like assemblies can either serve as individual drug carriers,29 or further entangle into a 3D network for use as hydrogels in various biomedical contexts such as protein, or cell delivery,164, 165 regenerative medicine,25, 166 tissue engineering,4, 162 cancer therapeutics,167 and immune therapies.138, 168 In all cases, one challenging yet important design consideration is the selective degradation of the resulting materials by enzymes/proteases of interest. This is because chemical breakdown presents the first step toward ultimate clearance of these synthetic materials once their duties are finished, and also because controlled degradation by relevant enzymes could play a critical role in regulating the release rate of the delivered cargo, or in the control of cell fates such as differentiation and migration when used as extracellular matrix mimics.91

One current strategy is to incorporate into the molecular design a particular functional group or peptide sequence that is specific to the targeted enzymes or proteases. Although this strategy works fairly well in the design of enzyme-triggered molecular assembly into well-defined nanostructures (catalytic reactions occurring on the unassembled molecules),169-171 only limited successes have been reported on the
enzyme-induced morphological transitions\textsuperscript{172} and the specific degradation of self-assembled supramolecular filaments (catalytic reactions affecting the assembled nanostructures).\textsuperscript{100, 102, 173-175} In the latter cases, it is not clear whether changes in the assembled structures (both morphological transition and nanostructure degradation) stems from the cleavage reactions occurring on the nanostructures, or on the individual, unassembled molecules which leads to dissociation of the assembled structures into cleavable monomers. Specific cleavage of assembled nanostructure often proves to be challenging, mainly because enzymatic reactions in biological systems often proceed with high precision, involving specific interactions of the enzyme with its substrate in the monomeric form. These specific interactions, however, can be greatly hindered, or even prohibited once the substrates assemble into supramolecular nanostructures. Inspired by the work of Wooley and coworkers on shell-crosslinked polymeric micelles\textsuperscript{176, 177} and also the work by Xu, Epstein, and coworkers on post-assembly crosslinking peptide nanofibers,\textsuperscript{178, 179} we report herein a crosslinking strategy to construct supramolecular filaments that can specifically break down in the presence of matrix metalloprotease-2 (MMP-2). Notably, we show that the cleavage reaction actually takes place more efficiently on the degradable linkers that joint the assembled nanostructures, leading to the breakdown of crosslinked filaments.

MMPs are extracellular proteases that regulate a variety of physiological processes and represent key players in facilitating molecular communication between cells and their surroundings.\textsuperscript{98} Recently, there has been a rapidly growing interest in the development of peptide-based supramolecular filaments responsive to specific MMPs
due to their important biomedical applications.\textsuperscript{100, 102, 172, 175} Examples include the RADA peptides containing MMP-2 specific substrates by Chau et al.,\textsuperscript{175} the self-assembling multidomain peptides developed by the Hartgerink lab (responsive to MMP-2),\textsuperscript{100} and the self-assembling $\beta$-hairpin degrading peptides developed by Schneider and coworkers (responsive to MMP-13).\textsuperscript{102} These researchers have shown compelling evidence that the resultant supramolecular hydrogels can break down in response to the cleavage reactions of their respective, targeted MMPs. However, since in these examples the MMP-specific sequences were incorporated into the molecular building units, their contributions to the self-assembly process could lead to difficulties in predicting the assembled morphologies as well as the mechanical properties of the resulting hydrogels. Using these systems for a different type of MMPs would require a complete redesign and characterization of the assembly system. Moreover, the formation of enzyme-substrate complexes, a critical intermediate step to facilitate the enzymatic reaction, is affected or even suppressed as the peptide substrate could be deeply embedded inside the respective nanostructures, inaccessible to its targeted MMPs. It is certain that molecular assembly into supramolecular nanostructures would complicate the specificity of the incorporated sequence to the targeted MMPs, most likely reducing the degradation rate. Therefore, it would be advantageous to design a peptide assembly platform that could separate the assembly process (structural and morphological control) from the incorporation of MMP degradation features into supramolecular filaments (MMP responsiveness).
5.3 Experimental Procedures

5.3.1 Peptide Synthesis.

All peptides used in this work were synthesized utilizing standard Fmoc solid-phase peptide chemistry on an AAPPTec Focus peptide synthesizer. In brief, in each amino acid coupling cycle, the resin first underwent Fmoc-deprotection through treatment with 20% 4-methylpiperidine in dimethylformamide (DMF), followed by reaction with a mixture of Fmoc-amino acid, O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), and diisopropylethylamine (DIEA) (4:4:6 mole equiv. relative to resin) in DMF. Side chain modification of the branching lysine was performed by use of an orthogonal protecting group strategy: the ε-amine group on the branching lysine was initially protected by 4-methyltrityl (Mtt) group that can be deprotected by 3% trifluoroacetic acid (TFA), 5% triisopropylsilane (TIS) in dichloromethane (DCM), and then reacted with a mixture of acid/HBTU/DIEA (4:4:6) in DMF. The N-termini for all designed peptides were acetylated using 20% acetic anhydride in DMF. The cleavage of the peptides from the resin was accomplished by treating the resin with 95% TFA, 2.5% TIS, 2.5% H₂O for 3 h. Cleaved peptides were isolated by removal of the solvents and precipitation with cold diethyl ether. Purification of the peptides was performed on a preparative HPLC column using a gradient of water and acetonitrile both containing 0.1% TFA or ammonium hydroxide. Fractions were collected and analyzed by matrix assisted laser desorption-ionization (MALDI-ToF) mass spectrometry. Product-containing fractions were combined and
lyophilized. All lyophilized peptides were stored in a -20 °C freezer. A detailed synthesis scheme can be found in Scheme S1.

5.3.2 Crosslinking

The self-assembling peptide MASP1 was first dissolved in 0.1 M MES buffer (pH 4.5) and allowed to form a gel overnight before the post-crosslinking treatment was carried out. The MASP1 solution (3.5 mM, 90 μL) was initially activated by the addition of EDC/NHS (mM, 10 μL) that was prepared in 0.1 M MES buffer (pH 4.5). The solution was vortexed for 5 s on the constant speed vortex mixer and settle for 10 min. To this solution added with peptide crosslinkers or polyamines (25.2 mM for PCLs and spermines, 50 mM for EDBE, 50 μL) that were prepared in 0.1 M MES buffer, and continue the reaction for 2 h at room temperature after vortexing for 5 s. The final stoichiometry was made up to 25-times excess for EDC/NHS, and 4-times excess for peptide crosslinkers or polyamines; the final concentration of MASP1 was 2.1 mM. All solution added to MASP1 solutions were adjusted to pH 4.5 by 1 M HCl prior to mixing. After 2 h of crosslinking, solutions were transferred into a dialysis unit against 1-L of deionized water to remove the side products and salts (MWCO 2000, Thermo Scientific) for 24 h. The solution pH was checked by pH test paper and adjusted to ~7 with 1 M NaOH if required.

5.3.3 Circular Dichroism

MASP1 was dissolved in 0.1M MES buffer (pH 4.5) or Tris buffer (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 100 mM CaCl2) at a concentration of 400 μM. The solutions were loaded onto a 0.1mm detachable cuvette and the spectra recorded on a JASCO
The mean residue ellipticity $[\theta]$ was calculated using the following equation

$$[\theta] = \frac{\theta}{c \times l \times n}$$

where $\theta$ is the measured ellipticity in mdeg, $c$ is the concentration in dmol L$^{-1}$ of the peptide calibrated by the absorbance of the tyrosine residue at 275 nm (1390 AU mol$^{-1}$ cm$^{-1}$), $l$ is the light path length of the cuvette in cm, and $n$ is the number of amino acid residues.

5.3.4 Transmission Electron Microscopy

Specimens for TEM were prepared by loading 5 $\mu$L of solution on a copper grid covered with carbon film and negatively stained by 2% uranyl acetate (Electron Microscopy Sciences, USA). Cryogenic TEM specimens were prepared by Vitrobot (FEI, USA): in brief, solutions were first loaded on grids with lacey carbon film (Electron Microscopy Sciences, USA), blotted by filter paper and transferred into liquid ethane; samples were kept in liquid nitrogen before imaging. The specimens were imaged on FEI Tecnai 12 TWIN electron microscope at 100 kV. The micrographs were acquired using a SIS Megaview wide angle camera.

5.3.5 Enzymatic Degradation Studies

Crosslinked networks were treated with active MMP-2 (Merck Millipore, USA; active, human, recombinant, CHO cells) in testing buffer (final concentration was made up to 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 100 mM CaCl$_2$) and incubated at room temperature for 6 h. Solutions were then loaded onto the stainless steel plate and mixed
with 1% sinapic acid for MALDI-ToF mass spectrometric analysis. The corresponding TEM specimens for degradation studies were prepared by loading 3 µL of tested solution on a copper grid covered with carbon film and negatively stained by 2% uranyl acetate.

5.4 Results and Discussions

Molecular Design. Figure 5-1 shows the rational design of the studied molecules illustrating two key design principles: 1) the reversible nature of the assembly–disassembly process (Figures 5-1a–b) and 2) the crosslinking of the assembled filaments using MMP-degradable sequences (Figure 5-1c). Our design rationale is to separate the molecular assembly event from the enzymatic cleavage reaction, with the aim of using the enzymatic cleavage to trigger the disassociation of the supramolecular filaments into individual building units. To accomplish this, we first create an amphiphilic peptide that has the ability to only self-assemble into 1D supramolecular filaments under acidic conditions (pH 4.5), where the crosslinking strategy can be applied to covalently lock the structure. These crosslinked filaments should then be stable under neutral conditions (pH 7.5) but will have the ability to quickly disassociate into individual molecules once the crosslinkers have been degraded by the targeted MMP.
Figure 5-1. Design principle of supramolecular filaments for controlled enzymatic degradation. (a) Chemical structure of the studied molecule and (b) the key design feature of the molecular assembly principle: the reversible nature of the self-assembled 1D filaments in response to pH variation. (c) Illustration of the crosslinking strategy and the expected degradation pathway by targeted MMP. PCL represents peptide crosslinkers (PCL) containing MMP specific substrates. The designed amphiphilic peptide is expected to self-assemble into stable 1D nanostructures at a lower pH value, followed by a post-crosslinking process that employs EDC/NHS chemistry and utilizes PCLs as linkers. Chemical structures of the PCL linkers are presented in Scheme 5-1.
Scheme 5-1. Chemical structures of the four crosslinkers designed/used in the study.

The design of the self-assembling peptide was based on a miktoarm star motif with each arm possessing a specific function necessary to imbue the desired assembly properties. Figure 5-1a shows the synthesized peptide MASP1, comprising three structural units: a β-sheet forming sequence plus three glutamic acid residues, an oligoproline segment, and a hydrophobic hydrocarbon. First, the peptide sequence of GNNQQQNY was chosen to achieve the desired one-dimensionality. This seven-residue peptide is derived from the key amyloid-forming region of the yeast prion Sup35, known to adopt a parallel β-sheet secondary structure in the native protein. Three glutamic acid residues were placed at the C-terminal of the peptide to impart the amphiphilic nature and the pH-responsiveness.

Second, the oligoproline is included to serve as a hydrophobic segment and, more importantly, is a key design feature to regulate the transition kinetics between the assembled and unassembled states. Oligoprolines are known as “molecular rulers”
because of their high propensity to form the stable polyproline type II (PPII) helical secondary structure.\textsuperscript{181, 182} It has been reported that when packed in the crystal form, the center-to-center distance between PPII helices is \textasciitilde6.6 Å,\textsuperscript{183} a distance greater than the characteristic hydrogen bonding length in \(\alpha\)-sheets (~4.7 Å). Consequently, if intermolecular hydrogen bonding between Sup35 segments occurs, the oligoproline segment must distort its natural PPII conformation to accommodate \(\beta\)-sheet formation (\textbf{Figure 5-2b}). We expect that this deformation of the PPII helical structure and its desire to return to its natural state will greatly accelerate the disassociation kinetics. Lastly, a short hydrocarbon tail (\(\text{C}_8\)) was introduced to provide increased hydrophobicity, enhancing the assembly potential of the designed molecules which were found to have poor assembly characteristics in its absence (vide infra). Details of the molecular synthesis, purification and characterization are described in the Materials and Methods section and in the Supporting Information [SI] (\textbf{Figures S5-1–S5-5} in SI).

\textbf{Characterization of assemblies.} The supramolecular filaments formed in MES buffer (~pH 4.5) can be clearly visualized using both transmission electron microscopy (TEM) (\textbf{Figure 5-2a}) and cryogenic-TEM imaging techniques (\textbf{Figure 5-2b}). The diameter of the observed filaments is approximately 10 nm, with lengths on the order of tens of micrometers. Macroscopically, MASP1 forms a clear, self-supporting gel above 2 mM in MES buffer but presents as a fluidic liquid in Tris buffer (pH=7.5) (\textbf{Figure 5-2c}). The gel-to-solution transition was found to occur instantly when the solution pH was raised from 4.5 to \textasciitilde7.0 by the addition of NaOH. TEM imaging showed no evidence of filamentous nanostructures existing in solutions aged overnight (\textbf{Figure S5-6} in SI).
Thioflavin T assay was used to confirm this self-assembling behavior that is responsive toward pH. Thioflavin T is a dye that exhibits enhanced fluorescent intensity when it binds to highly ordered amyloid structures. After incubating the Thioflavin T with MASP1 in MES and Tris buffer respectively, the dye fluoresced more intensively when mixed with MASP1 in MES buffer, where the filamentous structures were affluent in the solution; on the other hand, fluorescence of Thioflavin T was mostly quenched when mixed with MASP1 in Tris buffer (Figure 5-2d).

Figure 5-2. Characterizations of the designed peptide MASP1. (a) Chemical structure of the studied molecule and (b) the key design feature of the molecular assembly principle: the reversible nature of the self-assembled 1D filaments in response to pH variation. (c) Illustration of the crosslinking strategy and the expected degradation pathway by targeted MMP. PCL represents peptide crosslinkers (PCL) containing MMP specific substrates. The designed amphiphilic peptide is expected to self-assemble into stable 1D nanostructures at a lower pH value, followed by a post-crosslinking process that employs EDC/NHS chemistry and utilizes PCLs as linkers. Chemical structures of the PCL linkers are presented in Scheme 1.
Circular dichroism (CD) studies on the MASP1 aqueous solutions exhibited typical absorptions of the polyproline II (PPII) helical conformation in both acidic and neutral conditions, as evidenced by a strong negative peak at 205 nm and a slightly positive peak around 225 nm (Figure 5-2e). This PPII helical conformation can be attributed to the oligoproline segment. Given the one dimensionality of the assembled nanostructures under acidic conditions, we speculate the formation of intermolecular hydrogen bonding among Sup35 segments but its existence cannot be confirmed via CD measurements as the β-sheet absorption was seemingly overwhelmed by the PPII signals. Wide angle X-ray scattering experiments were therefore performed, revealing the characteristic β-sheet spacing reflection (~4.7 Å) (Figure S5-7 in SI). The existence of β-sheets strongly suggests the PPII helix (~6.6 Å minimum packing spacing) must be distorted within the filaments to accommodate the typical 4.7 Å spacing required for β-sheet formation. The slight difference in CD absorption (Figure 5-2d) might arise from this distortion of the PPII helix packing, combined with contributions from the β-sheet absorption.
Figure 5-3. Characterizations of C₈-Sup35. a) Chemical structure of C₈-Sup35. b–c) Cryo-TEM (b) and TEM (c) micrographs of the filamentous structures formed by C₈-Sup35 in acidic conditions (pH 4.5) at 2.1 mM. d) TEM micrograph of the filaments formed by C₈-Sup35 in pH ~ 7 at 2.1 mM. All bars = 200 nm.

We believe that the buried stress associated with the PPII deformation provides the impetus to rapidly lead to the dissociation of filamentous structures at pH~7. This observation is in line with reported work that the addition of a polyproline segment to the C-terminus of a polyglutamine sequence inhibits the formation of aggregation-prone β-sheets. Indeed, removing the C₈ segment in our system, we found that the resultant conjugate (P₉-G-Sup35) was unable to assemble into any well-defined structures at pH 4.5, possibly due to the β-sheet inhibitive nature imposed by the PPII-like conformation adopted by the oligoproline segment (Figures S5-8 in SI). We also noted that the P₉-G-Sup35 conjugate is capable of forming filamentous structures, only when the solution pH was lowered below 3. In contrast, the conjugate possessing no oligoproline (C₈-
Sup35) can self-assemble into filamentous nanostructures at pH 4.5 (Figure 5-3a–c).
However, these filaments do not rapidly disassociate upon raising the solution pH to around 7. After aging overnight, TEM examination still showed the existence of filaments as the dominant morphology (Figure 5-3d). Therefore, both the hydrocarbon and oligoproline segments are critical elements in the design of supramolecular filaments that possess rapid pH responsive behavior between pH 4.5 and 7.5.

Filament crosslinking two MMP-2 peptide substrates, GPQG-IAGQ and IPVS-LRSG, were chosen for the design of the peptide crosslinkers PCL1 and PCL2 (the hyphen denotes the expected cleavage sites by MMP-2). Both sequences were reported to be degradable by MMP-2 with high specificity, with the latter yielding an ~8-fold higher value of $k_{cat}/K_M$ than the former.186 Two lysine residues were placed at each terminal (Scheme 5-1) to react with the multiple carboxylic groups of the terminal glutamic acids on MASPI. Polyamines such as ethylenedioxybisethyamine (EDBE) and spermine were used as control crosslinkers for comparison. All crosslinking experiments were carried out in the assembly conditions (0.1 M MES buffer, pH 4.5) using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as initiators. The ratio of amines to carboxylic acids was chosen to be 4:1. After chemical crosslinking, the solution pH was adjusted to neutral conditions by adding NaOH to evaluate if this crosslinking strategy could preserve the filament structure. Figure 5-4a-d shows TEM images of the crosslinked filaments at pH ~7, indicating that all four crosslinkers can successfully stabilize the supramolecular filaments. No apparent differences in morphology and length were observed between the crosslinked filaments
and the noncrosslinked ones formed at pH 4.5, except that the crosslinked filaments seemed to have a greater tendency to bundle. It has been shown that the valency and the spacer distance between two adjacent reactive amines within the crosslinker play an important role in the crosslinking efficiency, leading to formation of different assembled morphologies.\textsuperscript{187} However, under our experimental conditions (amine: acid = 4:1), the crosslinker identity did not appear to be a factor in the adopted morphology. Instead, the molecular design of MASP1 is the primary factor determining the assembled structures, suggesting that other crosslinkers specifically cleavable by different enzymes can also be used to stabilize the MASP1 filament without altering the assembly behavior of MASP1.

Figure 5-4. Characterizations of PCL-crosslinked MASP1. TEM micrographs of filaments after the post-crosslinking treatment and qualitative analysis of crosslinked filaments. TEM micrographs of crosslinked filaments by PCL1 (a), PCL2 (b), EDBE (c), and spermine (d) at pH ~7. All scale bars = 200 nm. (e) Analytical HPLC traces of crosslinked filaments by PCL1 (blue) and PCL2 (orange) (solid lines). In all cases, the corresponding peaks remain sharp and monodisperse in the chromatograms if EDC/NHS were not introduced to initiate the crosslinking chemistry (dashed lines).
The crosslinking chemistry at the molecular level was first verified using analytical HPLC (Figure 5-4e). Without the addition of EDC/NHS initiators, mixtures of PCLs with MASP1 were eluted as two sharp, narrow peaks (dashed chromatograms in Figure 5-4e). MASP1 was consistently eluted after 17 min, while PCL1 and PCL2 were eluted after ~12 min and ~14 min respectively. EDBE and spermine were not observable due to their lack of absorbance at 220 nm (Figure 5-4e). Addition of the EDC/NHS initiators led to a broadening of the MASP1 elution peak (solid chromatograms in Figure 5-4e), clearly suggesting the existence of multiple products resulting from the crosslinking reaction.

MALDI-ToF mass spectrometry was then used to identify the molecular weights of the resultant chemical species. We found that these products can be assigned to species with varying numbers of MASP1 and PCLs (Figure 5-5a–c, and Table S5-1 in SI). Since the molecular weights of EDBE or spermine were very small relative to that of MASP1, mass spectra of their crosslinked products only list the numbers of MASP1. These results also suggest that the crosslinking reaction did not transform the whole filament into one gigantic polymer. Actually, products containing large numbers of MASP1 and PCL were rarely observed in both MALDI and HPLC experiments. However, this lower degree of crosslinking is sufficient to stabilize the supramolecular filaments as TEM imaging shows that the crosslinked filaments are stable for at least five days at pH ~7 (Figure S5-10 in SI). Quantification of the relative amount of different crosslinked products was not possible as the peak intensities in MALDI do not necessarily correlate to the absolute amount of the molecules present.
Figure 5-5. Enzymatic degradation studies on the crosslinked filaments in the presence of MMP-2. MALDI-ToF mass spectra of crosslinked filaments by PCL1 (a), PCL2 (b), EDBE (c) before the enzymatic treatment (top spectrum), after the addition of Tris buffer (middle spectrum), and after 6 h of incubation with MMP-2 (bottom spectrum). Peaks are labeled with the (m,n) format, in which m represents the number of MASP1 molecules and n represents the number of the crosslinking molecules. (d) Schematic illustration of a representative pentamer of MASP1 linked with five PCLs and their possible degradation products after enzymatic cleavage of the PCLs.

MMP-2 degradation experiments. The enzymatic degradation of the crosslinked MASP1 filaments was investigated by MALDI-ToF mass spectrometry. After 6 h incubation with MMP-2 (50mM Tris, pH 7.5, 0.1M NaCl, 10mM CaCl2), multiple new signals were observed for both PCL1- and PCL2-crosslinked filaments (Figure 5-5a–c, and Table S5-2 in SI). The molecular weight difference between these new species and the original masses in the untreated samples is typically around 800 Da, a value close to the molecular weights of the hydrolysis products of both PCL1 and PCL2 if they were cleaved at the expected sites (expected molecular weights of the hydrolysis products of PCL1: 818.92 and 805.96 Da; and PCL2: 876.05 and 850.02 Da). For crosslinked MASP1 molecules containing odd numbers of PCL residues, their degradation products can be assigned in the form of (m,n), for example (2,2.5) (Figure 5-5d). However, one cannot distinguish if the signal (2,2) resulted from molecules containing two MASP1 linked by
two PCLs, or from molecules containing two MASP1 linked by one PCL plus two PCL degradation segments (Figure 5-5d). For filaments crosslinked by EDBE, it is difficult to discern if chemical degradation takes place due to the limited resolution of the MS data obtained. These experiments reveal clearly that the peptide crosslinkers can be specifically degraded by the target MMP-2 at the desired cleavage sites at the molecular level.

To confirm that MMP-2 degradation of the crosslinked filaments had the desired effect, TEM imaging was performed on PCL-2 crosslinked filaments that had been treated for 6 h with MMP-2 (Figure 5-6). We found that most filaments had already dissociated, with only the occasional observation of some filamentous structures. In the absence of MMP-2, these crosslinked filaments were found to be very stable and did not exhibit any noticeable time-dependent morphological variation (Figure 5-6). The stability of the crosslinked filaments, combined with the observation of much shorter filaments after 6 hr incubation with MMP-2, suggests that the MMP-2 cleavage reaction actually took place on the crosslinkers displayed on the nanofiber surfaces as shown in Figure 1c, not on the unassembled molecules in solution. It is very unlikely that the crosslinked filaments would first dissociate into monomeric forms which will then be degraded by MMP-2 given the structural stability of the crosslinked filaments.
Figure 5-6. TEM micrographs and schematic representation elucidating the enzymatic degradation on the crosslinked filamentous networks. (a) TEM micrographs for the PCL2-crosslinked (top panels) and EDBE-crosslinked (bottom panels) filaments after 6 hr incubation with MMP-2. The lengths of PCL2-crosslinked filaments were significantly reduced (top left panels) after incubation with MMP-2 for 6 hr, while the EDBE-crosslinked filaments did not reveal any noticeable changes in filament length (bottom left panels). Both PCL2-crosslinked (top right panels) and EDBE-crosslinked filaments (bottom right panels) are very stable in the absence of MMP-2, and did not display any noticeable change in filamentous structures 6 hr after Tris buffer was added. (b) Schematic illustration of degradation pathways of supramolecular filaments crosslinked by MMP degradable and non-degradable linkers.

5.5 Conclusions

In this work, we have reported a cross-linking strategy to construct MMP-degradable filaments formed by amyloid derived amphiphilic peptides. One key design feature is the incorporation of an oligoproline sequence that allows for rapid disassociation of the assembled β-sheet rich nanostructures upon degradation of the peptide cross-linkers. We also show that the peptide substrates displayed on the filament surfaces are still accessible for specific MMP cleavage. Notably, the use of four structurally different cross-linkers did not alter the assembled morphology, suggesting
the possibility of using other peptide cross-linkers for different types of enzymes. Because the assembly process is well separated from the incorporation of MMP degradability, peptide substrates of other proteases could be chosen as a potential cross-linker to stabilize the MASP1 filaments. The cross-linked filaments may find their use as individual carriers for drugs or imaging agents because of their improved stability during circulation and also of their enzyme induced instability once reaching their targets. Hydrogels of the cross-linked filaments could be potentially used as scaffolding materials for cell and protein delivery, or for studying cell differentiation and migration.

In order to develop peptide-based supramolecular filaments and hydrogels with degradation kinetics controlled by the targeted proteases (e.g., MMPs) for ultimate biomedical applications, two important issues need to be addressed: First, the specificity of the chosen peptide substrates to the targeted protease must be quantified experimentally. The two peptide substrates used in our work, one derived from natural collagen and the other screened from a peptide library, are highly specific to the MMP-2 in the soluble form, however, it is not clear how much their specificity is altered when displayed on the filament surfaces. Although our MALDI experiments reveal that the cleavage sites of both PCL1 and PCL2 remain unchanged in our studies, future work should be pursued to quantify the specificity by directly measuring the value of $k_{cat}/K_M$. The second important issue is the toxicity of all the chemicals involved in the cross-linking reactions. Our recent experiments reveal that, although the designed peptide MASP1 do not possess any potential toxicities to cells, the EDC/NHS initiators used in our studies are highly toxic. Therefore, these toxic initiators must be completely
removed via extensive dialysis. Alternatively, other cross-linking methods such as Michael-type addition, or enzyme-catalyzed reactions could be used to covalently fix the peptide supramolecular filaments. Despite these challenges, we believe that our reported system present a new platform for the design of enzymatically degradable materials, and this platform can be potentially extended to construct filaments responsive not only to other members of the MMP family, but also to other types of proteases or enzymes.

5.6 Acknowledgements

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6 Tuning the Viscoelastic Properties of Peptide-Based Scaffolds

6.1 Abstract

We report a strategy of controlling the local viscoelastic properties of self-assembled hydrogels formed by amphiphilic peptides through the conjugation of isomeric hydrocarbons to a β-sheet adopting peptide sequence, which is expected to alter the molecular packing in the hydrophobic core and further influence the mechanical properties at micron scale.

6.2 Introduction

Mechanical properties of materials are known as key features that would affect their functionality. Rigidity of scaffolds, for instance, is known as part of physical cues that can affect the cellular behaviors in three-dimensional (3D) cell culture. Such synthetic matrix can be effectively constructed by employing molecular self-assembly as the fundamental principal, where the building blocks assemble spontaneously through non-covalent interactions. This strategy especially highlights the connections between molecular design of building blocks and resultant properties of the supramolecular assemblies, thus providing the possibility to control the mechanical properties from the bottom-up approach. However, it should be noted that the mechanical properties of these self-assembled matrices are often the combinative consequences based on several factors, including the stiffness of the filaments, mesh...
size, and the concentration of salts (e.g., divalent ions). Each factor can contribute to the bulk rheological properties, and it is difficult to decouple the subsequent effect of each factor independently in many occasions. In this communication, we report a strategy to tune the local viscoelastic properties (at submicron scale) of filamentous networks, where we aim to alter the stiffness of assembled filaments and control the scaffold stiffness independently of variation of mesh size and salt concentration.

Self-assembled one-dimensional (1D) structures are assemblies characterized by 1D growth; their building blocks often possess chemical structures that intrinsically induce strong directional intermolecular interactions, such as hydrogen bonding or $\pi-\pi$ interactions. Such filamentous matrix is considered as a type of semi-flexible networks, and, in practice, the overall stiffness of such materials is often controlled by varying the bulk concentration of the monomers. Though increasing the concentration, indeed, usually enhances the mechanical strength of the matrix, this approach is often complicated by the simultaneous changes of the pore/mesh size and the change of filament stiffness (caused by bundling of filaments). It is then essential to develop an alternative approach to isolate the combinative effects of mesh size and other parameters.

Herein, we conjugate different isomeric hydrocarbons with different geometry to a peptide segment to imbue the overall amphiphilicity (Figure 6-1A). Additionally, the difference in atomic arrangements is expected to offer different molecular packing requirements within the hydrophobic environments, and further affect the resulting mechanical properties at micron scale. We previously reported a self-assembling,
amphipilic peptide conjugate that assembles into 1D supramolecular structures; it possesses a miktoarm star construct that includes a β-sheet adopting peptidic domain GNNQQNY (originated from the yeast prion Sup35), an oligoproline segment, and a hydrocarbon segment. In this work aiming to alter the molecular packing, the hydrocarbon segment is replaced by three isomeric hydrocarbons. The designed peptides MASP2, MASP3, and MASP4 carry cyclopentylpropionyl, a cyclohexylacetyl or a cycloheptanecarboxyl group respectively (Figure 6-1A; characterizations in Supporting Information [SI]). The three chosen isomers possess different ring size and are expected to offer a mechanism for altering the packing in the hydrophobic environments in the assemblies without causing differences in the surface chemistry (Figure 6-1B).

Figure 6-1. Main design rationale used in this study to tune the local viscoelastic properties of supramolecular polymer network through the use of isomeric peptides as building units. A) chemical structures of the designed peptides investigated in this study. B) estimation of the ring size of the cycloalkyl chain in the peptides of interest. Ring size in 3D space was estimated by MarvinSpace, ChemAxon.
6.3 Experimental Procedures

6.3.1 Peptide Synthesis and Purification

All peptides used in this work were synthesized by utilizing standard Fmoc solid-phase peptide chemistry with an AAPPTec Focus peptide synthesizer. In brief, in each amino acid coupling cycle, the resin first underwent a Fmoc-deprotection by treated with 20% 4-methylpiperidine in DMF, and was sequentially treated with activated Fmoc amino acid by using 4 Equiv of HBTU, 6 Equiv of diisopropylethylamine (DIEA). Side chain modification of branching lysine was done by using orthogonal chemistry: the e-amine group on the branching lysine was initially protected by Mtt group and can be deprotected by 3% TFA, 5% TIS in DCM, and then reacted with 4 Equiv of acid, 4Equiv of HBTU, and 6 Equiv of DEIA. The N-terminals for all designed peptides were acetylated by using 20% acetic anhydride in DMF. The cleavage of the peptides from the resin was accomplished by treating the resin with 95% TFA, 2.5% TIS, 2.5% H2O for 3 hours. Purification of the peptides was performed on a preparative HPLC column by using a gradient of water and acetonitrile both containing 0.1% of ammonium hydroxide. Purified liquids were collected and analyzed matrix assisted laser desorption-ionization (MALDI-ToF) mass spectrometry and then lyophilized. All lyophilized peptides were stored in a -20 C freezer.

6.3.2 Circular Dichroism

The CD spectra for all peptides were monitored by JASCO J715 spectropolarimeter in the far UV region (190-260 nm). Each solution was loaded in 0.1 mm detachable cuvette. The molar residual ellipticity was calculated by the following equation
where theta is the obtained ellipticity, c is the concentration of the peptide calibrated by the absorbance at 275 nm caused by the tyrosine residue (extinction coefficient = 1390), l is the light path length of the cuvette, and n is the number of amino acid residues.

6.3.3 Wide Angle X-Ray Scattering

2 wt% Diffraction data were obtained by APS (Advanced Photon Source) synchrotron radiation at the beamline BioCARS (14-BMC) in Argonne National Laboratory. The distance of sample to beamstop was 100 mm, while the sample to detector distance was 600 or 900 mm. Diffraction patterns were recorded by an AdSC Quantum315 CCD detector.

6.3.4 Transmission Electron Microscopy

The TEM specimens were prepared by loading 5 μL of peptide solutions on copper grids with supporting carbon film, while subsequently the excess liquids on the grid were initially removed by filter papers. The specimens were then loaded with another 5 μL of 2% uranyl acetate, and were blotted by filter papers to remove the excess uranyl acetate. TEM imaging were carried out after the specimens were air-dried on a FEI Tecnai 12 TWIN transmission electron microscope equipped with SIS Megaview III wide angle camera at 100kV.

6.3.5 Determination of Critical Aggregation Concentration

The determination of CAC was carried out by a previously reported protocol. In brief, a fixed content of Nile Red (1 μM) is used as the reporter to probe the presence of
micellar structures formed by the amphiphilic peptides in 0.1M MES buffer (pH 4.5) at various peptide concentrations after equilibrating overnight. Fluorescent spectra of Nile red were then monitored by a Fluorolog fluorometer (Jobin Yvon, Edison, NJ) with fixed excitation wavelength at 560 nm; spectra were monitored within 580–720 nm. The ratios of the emission intensity at 635 nm (near the emission maximum when Nile Red was encapsulated in the hydrophobic environments) to that at 660 nm (the weak emission maximum in aqueous conditions) were then plotted against the peptide concentrations. The CMC values were determined by calculating the crossover point of the ratio curve plotted against the peptide concentrations before/after the intensity surge.

6.3.6 Particle-Tracking Microrheology

Specimens used for microrheological studies were prepared by mixing lyophilized peptides with mini-Q water containing 0.0001% solids of polystyrene fluosphere (carboxylated, 1um, Invitrogen) as probes. The mixture of peptide solutions and fluospheres were immediately loaded into an 8-well glass-bottom plate before gelation, and aged for 3 days before any further analysis. Suspension of the fluorescent particles was tracked with high spatial and temporal resolutions using high magnification objective (60x Plan Apo lens, N.A. 1.4, Nikon Melville, NY). Movies of the Brownian motion of the fluorescent particles were taken at 30 frames per second for 20 seconds with an EMCCD camera (Andor Technology) mounted on a Nikon TE2000 microscope (Nikon Melville, NY) controlled by Nikon NIS-Element software. Quantification and post analysis of particle trajectories were done with the customized Matlab code to obtain rheological parameters to describe the viscoelastic properties of the hydrogels. At least
20 particles were tracked and analyzed per condition. The dynamics of embedded particles in a viscoelastic material can be described by the extension of Stokes-Einstein Equation,

\[ \langle \Delta r^2(t) \rangle = \frac{k_B T}{na} J(t) \]

where \( \langle \Delta r^2(t) \rangle \) is the time-averaged means squared displacement (MSD) of the embedded nanoparticles, \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature, \( a \) is the radius of the nanoparticles, \( J(t) \) is the creep compliance.

### 6.4 Results and Discussions

The resulting nanostructures in the materials were first visualized by transmission electron microscopy (TEM) and cryogenic-TEM (cryo-TEM) imaging. When dissolved in water (pH 4.5), all designed peptides self-assembled into long 1D, twisted-ribbon like filaments whose width ~20 nm, while the length could reach several microns after one day of incubation at 1% (w/v; 2.1 mM) (Figure 6-2). Circular dichroism (CD) spectra of the peptides reveal the secondary structures of the peptides and the intermolecular interactions. In line with our previous observation, the designed peptide showed strong characteristics (strong negative peak at 205 nm, very weak positive peak at 235 nm) of polyproline type II (PPII) helical structures because of the oligoproline segment incorporated in each molecule (Figure 6-3A). The characteristics of \( \beta \)-sheet conformation were not explicitly displayed in the CD spectra, potentially due to weaker signal intensity compared to the PPII-like conformation.
Notably, reflective peaks at ~4.7 Å, which matched the spacing of the hydrogen bonds of β-strands in the neighboring peptides, were observed in wide angle X-ray scattering (WAXS) for all designed peptides (Figure 6-3B–D). It was also noted that MASP3 and MASP4 showed a more diffusive peak at 4.7 Å; this peak broadening could be related to the smaller size of mean ordered domains in submicron scale. This phenomenon is very likely linked to how the molecular packing of monomers can hierarchically impact the behaviors of the soft matter at submicron scale: the larger the cycloalkyl group in the hydrophobic moiety, the more irregular the peptides packed. According to the observation in CD spectra and WAXS patterns, we concluded that peptide MASP2, MASP3 and MASP4 simultaneous exhibited the characteristics of PPII and β-sheet conformation, whereas the β-sheet characteristics were not strongly exhibited in the CD spectra due to the extremely strong signals from the PPII conformation.
We then examine the self-assembling requirements for the three design peptides. It is estimated that overall hydrophobicity of the isomeric peptides is similar, though they possess different hydrocarbons, since the elution time of each molecule in analytical RP-HPLC is almost the same (see SI). The critical aggregation concentration (CAC) value of each amphiphilic peptides was then measured by incubation of peptide solutions with a reporter dye Nile Red. Nile Red is a hydrophobic dye that exhibits enhanced fluorescent intensity and blue-shifted when exposed in hydrophobic environments. We mixed
each isomeric peptide solution (in MES buffer, pH 4.5) at varied concentrations with a fixed content of Nile Red, and then monitored the florescence spectra after overnight. The transition of blueshift indicates the Nile Red starts to be encapsulated in hydrophobic cores when the peptide concentration surpasses the CAC value, where the transition was determined by plotting the ratio of emission maximum of Nile Red in hydrophobic environments ($\lambda_{\text{max}}$: 635 nm) to that in aqueous conditions ($\lambda_{\text{max}}$: 660 nm) against the peptide concentrations. Our results suggest the CAC values of MASP2, MASP3, and MASP4 are similar; the CAC values lie in the range of $\sim$50 $\mu$M in pH 4.5. In self-assembly systems where the building blocks are small molecules, it is considered that there is a fast dynamic equilibrium of assembled structures and monomers; therefore, in our case, the peptides can exists as assembled filaments and monomers. Since the CAC values of MASP2, MASP3 and MASP4 are very similar, the actual content of assembled peptides will be almost the same given all solutions possess the same bulk concentration. In addition, our results from TEM imaging suggest there is no obvious morphological difference in terms of filament length and width. The spatial distribution of filaments is estimated to be very similar for all MASPs solution at the same bulk concentration; under this regard, the mesh size of the resultant hydrogels shall be very similar for all MASPs at the same bulk concentration.
Figure 6-3. Characterizations of studied peptides. A) CD spectra of MASP2-4. Spectra were monitored for 0.167% (w/v) solutions that were instantly diluted from 1% (w/v) solutions. B-D) WAXS patterns of the MASP2-4 at 2% (w/v). The reflective peaks at ~4.7 Å (indicated by the arrows) correspond to the spacing of intermolecular hydrogen bonds in β-sheet. E-G) Determination of the CAC values of peptides by Nile red encapsulation for MASP2 (E), MASP3 (F) and MASP4 (G); the transition of I635/I660 indicates the intensity surge of Nile Red when exposed in hydrophobic environments when the peptide concentration exceeds the CAC. The obtained CAC values for MASP2, MASP3, MASP4 are ~40 μM in 0.1M MES buffer (pH 4.5). Data are presented as mean ± standard deviation (n=3).

The local viscoelastic nature of the materials was then evaluated by using particle-tracking microrheology for all peptide solutions in mini-Q water (pH adjusted to 4.5 by hydrochloric acid). Unlike bulk rheology, particle-tracking rheology provides the
information about localized stiffness in the materials in micron to submicron scale.\textsuperscript{203-205} The local viscoelastic properties of solutions or hydrogels can be investigated by single-particle tracking: as particles are embedded in viscoelastic materials, the movement of the probing particles is governed by the fluctuation of biopolymer chains where the mean-squared displacement (MSD) of the embedded particles can be described by generalized Stokes-Einstein equation.\textsuperscript{205-207} In brief, the probing particles undergo unrestricted Brownian motion in viscous liquids and, thus, they can be directly correlated with Stokes law, suggesting the MSD is propositional to the time lapse and showing a slope of unity in the log-log plot of MSD against time lag. In contrast, the MSD of probing particles in elastic solids is completely independent of time and show a slope of zero in logarithmic plots. For viscoelastic materials, the MSD becomes sub-diffusive and follows a relation: 

\[ \langle \Delta r^2 \rangle \propto t^\alpha, \]

where \( \alpha \) is the logarithmic slope of MSD versus time lag.\textsuperscript{204-206} In the high frequency regimes (or short time lag regimes), the movement of the probing particles is governed by the lateral thermal fluctuation of the surrounding filaments as the particles are trapped within the filament networks.

As the molar concentration of each solution was identical (4.2 mM; 1 wt%), the ensemble-averaged MSD of the probing particles in the scaffolds showed the trend as: 

\[ \text{MSD}_{\text{MASP2}} < \text{MSD}_{\text{MASP3}} < \text{MSD}_{\text{MASP4}} \]

under all monitored time scales (Figure 6-4). The MSD of the probing particles did not divide into two independent populations, implying the mesh size of the networks is smaller than the size of the probing particles (1 \( \mu \)m) (Figure S6-5). We conclude that the larger the conjugated cycloalkyl group in the amphiphilic peptides, the larger the magnitude of the MSD. This phenomenon is indicative of
lowered local stiffness of resultant matrices if bulker hydrocarbon is designed in the molecule. Representative trajectories of an embedded particle also reveal the trends of viscoelastic characteristics of the networks, where the probing particles display larger displacement in a time span of 7 seconds while trapped in a relatively weaker hydrogel formed by MASP4 (Figure 6-4B). It is also noted the logarithmic slopes of MSD vs time lag, $\alpha$, of the probing particles lies in the range between zero and unity, suggesting the subdiffusive behaviors.

Figure 6-4. Microrheological analysis of the designed peptide hydrogels. A) Mean-squared displacement versus time lag probing particles embedded within 2.1 mM MASP2, MASP3 and MASP4. Data are presented as the ensemble MSD ± standard error of the mean. B) representative trajectories of the movement of the center of a single probing particle embedded within MASP2, MASP3, and MASP4 scaffolds. All bars = 1 μm.

The ensemble MSD of MASP4 possesses a $\alpha$ value that is very close to unity, showing the material behaves almost viscous like. Under the tested concentration (2% (w/v); 4.2 mM), all the materials formed by the peptides were below the concentration to surpass the sol-gel transition, since $\alpha$ is between 0.5 and 1 for all materials, which were
compromised by the optimization of sufficient signal-to-noise ratio in passive particle-tracking microrheology.

It is known that the difference of local viscoelastic properties is strongly linked to the bending and stretching fluctuation of the chain under high short time lag (high frequency) and long time lag (low frequency) respectively. Our results suggested the mesh sizes of the networks formed by MASP2, MASP3, MASP4 were similar at the same peptide concentration. Thus, the embedded particles are estimated to possess similar trapping conditions and reflect both bending and stretching behaviors of the assembled filaments. Under all monitored time span, the microrheological measurements showed a trend where the stiffness of the networks can be correlated with the regularity of molecular packing as evident by WAXS: the more diffusive of the reflective peak in scattering pattern, the weaker the mechanical strength of the matrix. The origin of this behavior can be further traced down to the molecular design, where the size of the ring of the isomeric hydrocarbons would alter the molecular packing within the hydrophobic environments.

6.5 Conclusions

In summary, we have developed a platform to construct 3D matrices and harness the local viscoelastic properties that originated from the bottom-up design of synthetic peptides, while the other material parameters remained unchanged. Since such peptide based hydrogels are relatively clean systems for further applications compared with many naturally occurring matrices, the fine-tuning of the local mechanical properties
will be highly effective if these synthetic materials are applied in cell culture. We believe this design rationale could be an effective means to construct synthetic materials with an aim to elucidate or differentiate the roles of local mechanical properties and geometric sensing in directing cell behaviors with controlled surface chemistry and mesh size.

6.6 Acknowledgements

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7 Conclusions and Future Work

Peptide-based materials are effective building units for fabricating supramolecular polymers while they can easily assemble into 1D assemblies by properly designing the amino acid sequences. The materials formed by such bottom-up strategy, whose function can be easily incorporated by assigning specific functional groups within the building blocks, provide potential applications in drug delivery and regenerative medicine. Understanding the critical roles of design parameters of peptide-based building blocks provides a basis for control the morphology and function of the assemblies that could be applied in the developments of effective therapeutic cargos as well as functional scaffolds. In particular, this work discovers the key design and factors that direct the supramolecular polymerization into different types of 1D structures, and strategies for controlling the function, enzymatic degradation and rheological properties.

First, this platform can further be extended to design filamentous networks that formed by other types of therapeutic, not being limited to chemotherapeutics. For instance, ketoprofen (a nonsteroidal anti-inflammatory drug) and sulfamethazine (a sulfonamide antibacterial) can both be easily conjugated to a peptide segment via simple chemical syntheses. The conjugation of these different types of such therapeutics into a β-sheet adopting peptide sequences allows the resultant drug amphiphiles assemble into filaments (Figure 7-1), which could attribute to the development of functional hydrogels with either anti-inflammatory or antimicrobial properties.
Figure 7-1. Examples of supramolecular polymers formed by drug amphiphiles conjugated with ketoprofen or sulfamethazine. Chemical structures of designed DA possessing a ketoprofen (a) or a sulfamethazine (b) segment, and the TEM micrographs of their resulting 1D assemblies in aqueous media. TEM micrograph (a) credit: Ran Lin.

Secondly, the construction of nanotubes, introduced by using catanionic mixtures or by incorporating metal coordinating groups as fabrication means, also offers great potential for development of organic conductive materials. Though it is not specifically discussed in this work, self-assembled nanotubes possess various promising and interesting applications in the energy fields. Being strongly characterized by \( \pi-\pi \) interactions, the systems discussed in Chapter 2 & 3 offer an extremely effective design rationale and strategies that could also be included when designing nanotubes as nanowires with diameters at the order of hundreds of nanometers.

Finally, the combination of miktoarm construct and isomeric peptides in designing building blocks offers a flexible platform to control of local viscoelastic properties of different types of functional scaffolds. An important feature of this proposed methodology is that the surface chemistry and requirements of self-assembly are very
similar among the isomeric peptide conjugates, which could assist elucidating the particular role of local viscoelastic properties in further applications without the simultaneous complication of other factors. Since mechanical properties of 3D scaffolds present as important cues in affecting the behaviors of embedded cells, it is absolutely interesting to discover the subjected behaviors of cells when they are cultured within different matrices.
Supporting Information for Chapter 2

Scheme S2-1. Chemical structures of mCPT-Sup35-K₂, mCPT-Sup35-E₂, dCPT-Sup35-K₂, and dCPT-Sup35-E₂.

Figure S2-1. RP-HPLC (a) and ESI-MS (b) characterization of qCPT-Sup35-K₂. m/z 1926.88 [M+2H]²⁺, 1292.06 [M+Na+2H]³⁺

Figure S2-2. RP-HPLC (a) and ESI-MS (b) characterization of qCPT-Sup35-E₂. m/z 978.4 [M+H+3Na]⁴⁺.
The drug loading of the DAs is calculated by the following equation

\[
\text{Drug loading} = \frac{nM_{\text{CPT}}}{M_{\text{DA}}} \times 100\%
\]
where $n$ is the number of CPT molecules conjugated, $M_{\text{CPT}}$ is molecular weight of CPT (347.1 g/mol), and $M_{\text{DA}}$ is the molecular weight of the DA.

\[
\text{qCPT-Sup35-K} \ _2 \ drug\ loading = (4 \times \frac{347.1}{3851.4}) \times 100\% = 36\%
\]

\[
\text{qCPT-Sup35-E} \ _2 \ drug\ loading = (4 \times \frac{347.1}{3854.2}) \times 100\% = 36\%
\]

\[
\text{dCPT-Sup35-K} \ _2 \ drug\ loading = (2 \times \frac{347.1}{2364.6}) \times 100\% = 29\%
\]

\[
\text{dCPT-Sup35-E} \ _2 \ drug\ loading = (2 \times \frac{347.1}{2367.5}) \times 100\% = 29\%
\]

\[
\text{mCPT-Sup35-K} \ _2 \ drug\ loading = (2 \times \frac{347.1}{1685.8}) \times 100\% = 21\%
\]

\[
\text{mCPT-Sup35-E} \ _2 \ drug\ loading = (2 \times \frac{347.1}{1688.7}) \times 100\% = 21\%
\]

According to our previous studies, it was noted \textbf{qCPT-Sup35} and its analogue (when the peptide segment was replaced by another $\beta$-sheet adopting sequence) spontaneously assemble into nanotubes (Figure S2-7). The nanotubes reported in our previous work possess a diameter of ~12 nm and a hydrophobic channel ~ 2 nm in aqueous media. It should be noted the previously described nanotubes are very different form the nanotubes formed by CAMs in terms of their dimensions. The “unexpected” observation of nanofilaments rather than nanotubes is the subject of ongoing investigations in our laboratory. Preliminary data suggests this interesting behavior is a kinetic phenomenon resulting from the HFIP treatment disrupting preformed nanostructures and will be reported in due course. CD spectra of the solutions prepared by these two different pathways also exhibit different patterns, suggesting the molecular packing that results to the two distinctive morphology are also different (Figure S2-8).
Figure S2-7. TEM micrographs of qCPT-Sup35-K2 (a) and qCPT-Sup35-E2 (b) in H2O after one-day of aging, where the solutions were previously lyophilized in 1:1 MeCN/H2O. This sample preparation will lead to the formation of nanotubes rather than filaments. The dark lines in each 1D structure were the results of staining by uranyl acetate after the collapse of tubular structures, being indicative of the channels of the tubular construct. Concentration = 400 μM. All bars = 200 nm.

Figure S2-8. CD spectra (solid lines) and UV-Vis spectra (dashed lines) of qCPT-Sup35-K2 (a) and qCPT-Sup35-E2 in H2O. Spectra were obtained from 400 μM solutions that were diluted to 50 μM immediately prior to measurement.
Figure S2-9. Cryo-TEM and TEM micrographs of the CAMs of qCPT-Sup35. Undulation of tubule size at the overlapping area of a tubule and the lacey carbon film (a), clusters of belt/sheet-like structures (b), and a multi-walled tubule (c) in a 100 μM solution of qCPT-Sup35 CAM 1:3. (d–f) TEM micrographs of qCPT-Sup35 CAM with mixing ratios of 1:3 (d), 1:1 (e) and 3:1 (f) at 400 μM in 1:1 MeCN/H2O. All bars = 500 nm.

Figure S2-10. CD spectra (solid lines) and UV-Vis spectra (dash lines) of CAMs of qCPT-Sup35 with a mixing ratio of 1:1 (a) and 3:1 (b) in 50% MeCN/H2O. The CD spectra for 50 μM solutions that were recorded immediately after dilution from 400 μM solutions in 50% MeCN/H2O. UV spectra were monitored for stock solutions made to 400 μM in 1:1 MeCN/H2O.
Figure S2-11. TEM micrographs of dCPT-Sup35-K₂ (a), dCPT-Sup35-E₂ (b), and CAMs of dCPT-Sup35 with a mixing ratio of 1:3 (c), 1:1 (d), or 3:1 (e). All samples revealed the filamentous structures while the bulk concentration was 400 μM in 1:1 MeCN/H₂O. Specimens were negatively stained by 2% uranyl acetate. All bars = 200 nm.

Figure S2-12. CD spectra of the CAMs of dCPT-Sup35 (a) and individual DAs in the system (b). The spectra were monitored for 100 μM solutions that were immediately diluted from 400 μM solutions in 1:1 MeCN/H₂O.
Figure S3-1. ESI mass spectra of N-succDFO (a) and crude materials of N-succDFO-Cys-NH₂. Calculated exact mass of N-succDFO: 660.37; [M+H]^+ 661.2; [M+Na]^+ 683.4; [M+K]^+ 707.4; [M-3H+Na+Fe]^+ 736.3. Calculated exact mass of N-succDFO-Cys-NH₂: 762.39; [M+H]^+ 763.3

Figure S3-2. $^1$H NMR spectra of HO₂C-buSS-Pyr.
Figure S3-3. $^1$H NMR (a) and MALDI-ToF mass spectra (b) of CPT-buSS-Pyr. Calculated exact mass: 559.12; [M+H]$^+$ 560.079; [M+Na]$^+$ 582.079.

Figure S3-4. ESI mass spectrum (a) and RP-HPLC chromatogram (b) of DFO-C-CPT. Calculated exact mass 1210.50; [M+H]$^+$ 1211.3; [M+Na]$^+$ 1233.5; [M+K]$^+$ 1249.5 [M+H+K]$^{2+}$ 625.2.
Figure S3-5. (a–b) TEM (a) and cryo-TEM (b) micrographs of the nanotubes formed by DFO-C-CPT in 1 equiv Fe(III). (c) Coexistence of filaments and nanotubes formed by DFO-C-CPT in 0.25 equiv Fe(III). (d) Observation of multiwalled construct in cryo-TEM imaging. White arrows indicate the stronger contrast resulted from the multiwalled nanotubes compared with nanotubes that possess less stacked layer(s) (indicated by black arrows). (e) Multiwalled nanotubes with enlarged tubule thickness. Regular TEM specimens were negatively stained with 2% (w/v) uranyl acetate. All specimens were prepared from a 500 μM DFO-C-CPT solution.

Figure S3-6. Observed intermediate structures toward tubular formation in a 500 μM DFO-C-CPT solution. (a–c) Representative TEM micrographs of helical ribbons (a–b, indicated by white arrows) and ribbon-wrapped nanotubes (c). All TEM specimens were negatively stained with 2% (w/v) uranyl acetate.
Figure S3-7. Circular dichroism (CD) spectra of DFO-C-CPT in H₂O (a) and 1 equiv Fe(III) (b) upon sequential dilution. The spectra were measured after instant dilution from a 500 μM solution that has been aged overnight, following up by stepwise dilution (2X per step), and loaded in a 2-cm cuvette.

Figure S3-8. (a) Release kinetics of 50 μM DFO-C-CPT in PBS or PBS with 1 equiv Fe(III) (mol relative to the DA) in the presence/absence of glutathione (GSH). Data were presented as mean ± stdv (n = 3) (b–c) Representative sequential RP-HPLC traces of DFO-C-CPT in PBS (b) or in PBS/1 equiv Fe(III) (c) when incubated with 10 μM GSH, which leads to the breakdown of disulfide bonds and facilitates the hydrolysis of ester bond between the camptothecin and buSS linker. (d–e) Representative sequential RP-HPLC traces of DFO-C-CPT in PBS (d) or in PBS/1 equiv Fe(III) (e) without the absence of GSH.
Supporting Information for Chapter 4

Figure S4-1. MALDI-ToF mass spectrum (A) and analytical RP-HPLC trace (B) of FHP. [M+Na]+ 1745.399; calculated exact mass: 1722.62

Figure S4-2. MALDI-ToF mass spectrum (A) and analytical RP-HPLC trace (B) of FP. [M+Na]+ 1661.068; [M+K]+ 1677.573; calculated exact mass = 1638.53.

Figure S4-3. MALDI-ToF mass spectrum (A) and analytical RP-HPLC trace (B) of HP. [M+Na]+ 1413.270; [M+K]+ 1429.243; calculated exact mass: 1390.64.
Figure S4-4. Emission spectra of the reporter dye Nile Red when incubated with various concentrations of peptides FHP (A), FP (B) and HP (C), for determining the critical micellation concentration (CMC) values. All spectra shown here are normalized by the emission maximum, and display a blueshift when the peptide concentrations surpass the CMC.

Figure S4-5. TEM and cryo-TEM micrographs of a 0.1% FHP solution after aging. (A–C) The twisted ribbons (A), intermediate structures between twisted ribbon and helical coil (B, transition was indicated by arrows) formed by FHP after 2 months of aging. (C–D) Cryo-TEM micrographs for a 0.1% FHP solution after 2 months of aging. The arrow in panel E indicates a narrow helical coil. (E–G) TEM micrograph of 0.1% FHP after 4 months (E) and 6 months (F, G) of aging, revealing various complex structures including twisted ribbons, type I helical ribbons, and type II helical ribbons (H–I) Representative cryo-TEM micrographs of a 0.1% FHP solution after 6 months of aging. All regular TEM specimens were negatively stained by 3% uranyl acetate. Scale bars = 200 nm.
Figure S4-6. (A) Schematic representation of the instrumentation for topological analysis. The projections in TEM imaging were acquired while the sample grid was tilted, revealing the three-dimensional topology of assembled structures. (B–C) TEM micrographs of helical ribbons (type I) while the tilted angle was set to $-\alpha$ (left), 0° (middle), and $\alpha$ (right). $\alpha = 30$ and 45 in panel B and C respectively. Samples were prepared from a 0.1% FHP solution that has been aged for 2 months. (D–F) Tapping mode AFM analysis of the 1D structure formed by a 0.1% FHP solution after 6 months of aging.

Figure S4-7. TEM micrographs of the supramolecular filaments formed by FP (A) and HP (B) at 0.1% after 2 months of aging. All bars = 200 nm.
**Scheme S4-1.** Chemical structure of H₃P.

**Figure S4-8.** MALDI-ToF mass spectrum (A) and analytical RP-HPLC trace (B) of H₃P. [M+H]+ 1497.724; [M+Na]+ 1513.708; calculated exact mass: 1474.73.

**Figure S4-9.** Representative TEM micrographs of H₃P at 0.1% in H₂O. Samples were stained by 3% uranyl acetate. All bars = 200 nm.
Supporting Information for Chapter 5

Figure S5-1. Synthesis route for MASP1.

Figure S5-2. Analytical RP-HPLC chromatogram (a) and MADLI-ToF mass spectrum of MASP1.
Figure S5-3. Analytical RP-HPLC chromatogram (a) and MADLI-ToF mass spectrum of P9-G-Sup35.

Figure S5-4. Analytical RP-HPLC chromatogram (a) and MADLI-ToF mass spectrum of C8-Sup35.

Figure S5-5. Analytical RP-HPLC chromatogram (a) and MADLI-ToF mass spectrum of PCL1.

Figure S5-6. Analytical RP-HPLC chromatogram (a) and MADLI-ToF mass spectrum of PCL2.
Figure S5-7. a-d, TEM micrographs of uncrosslinked MASP1 when pH was adjusted from 4.5 to ~7 by adding NaOH. Samples were prepared from solutions of MASP1 (a), MASP1+PCL1 (b), MASP1+PCL2 (c), and MASP1+spermine (d). TEM imaging shows no evidence of filamentous nanostructures or any other types of well-defined nanostructures at pH ~7. The observed objects in the above TEM images are likely a result of sample drying or staining and do not suggest the existence of such dominant aggregates. These TEM results imply the instability of uncrosslinked MASP1 filaments at pH ~7 and their quick dissociation into unassembled forms. All bars = 500 nm.
Figure S5-8. Wide angle X-ray scattering (WAXS) pattern collected from a 2% MASP1 aqueous solution (by weight). The doublet peaks (indicated by white arrows) correspond to Bragg spacing of 4.59Å and 4.76Å that can be attributed to the scattering from the intermolecular hydrogen bonding among Sup35 segment, a signature of β-sheet secondary conformation. Diffraction data were obtained by the Advanced Photon Source synchrotron radiation at the beamline BioCARS (14-BM-C) at the Argonne National Laboratory. The distance of the sample to the beamstop was set to 100 mm; sample to detector distance was fixed at 600 mm. Diffraction patterns were recorded by an ADSC Quantum315 CCD detector.

Figure S5-9. Emission spectra of Nile red when equilibrating with MASP1 in MES buffer (pH 4.5) and Tris buffer (pH 7.5). MASP1 concentration: 2.1 mM; Nile red concentration: 1 μM; excitation wavelength: 550 nm.
We synthesized two control molecules (P9G-Sup35 and C8-Sup35) to help understand the assembly behavior of MASP1. The chemical structure of P9G-Sup35 is identical with that of MASP1, except for replacement of the octyl group with an acetyl (Fig. S5-8a). P9G-Sup35 shows a high propensity to adopt the PPII secondary conformation (Fig. S5-6d) and demonstrates a poor ability to assemble into well-defined structures at pH 4.5, on the basis of our results from WAXS experiments (Fig. S5-9b) and TEM imaging studies (data not shown). We also noted that P9G-Sup35 possesses the ability to self-assemble into filamentous nanostructures only when the solution pH was lowered to around 3 to largely protonate the carboxylic acids of the glutamic acid residues. The chemical structure of C8-Sup35 is identical with that of MASP1, except for replacement of the oligoproline segment with an acetyl (Fig. S5-9a). In contrast to P9G-Sup35, C8-Sup35 molecule self-assembles into filamentous nanostructures at pH 4.5, and both CD (Fig. S5-9e) and WAXS (Fig. S5-9c) confirm the existence of the β-sheet secondary structures. Notably, the filaments formed by C8-Sup35 are rather stable in PBS buffer (pH 7.4) or in water with an adjusted pH~7.0.

Conclusion

For P9G-Sup35, the CD study reveals that the P9G-Sup35 molecule exhibits a PPII helical secondary structures at pH 4.5, and the WAXS spectrum shows no signs of β-sheet assemblies. For C8-Sup35, the CD study reveals that the C8-Sup35 molecule exhibits a typical β-sheet conformation, and this observation is further supported by the observed typical β-sheet X-ray reflections in WAXS. These experiments suggest that the existence of PPII helices hinders the formation of β-sheet assemblies even at pH 4.5 due
to the discrepancy in packing distance (6.2 Å for PPII helices vs. ~4.7 Å for peptides in β-sheets)

**Figure S5-10.** a, Chemical structures of P9G-Sup35 and C8-Sup35. b-c, WAXS scattering patterns of P9G-Sup35 (b) and C8-Sup35 (c) at pH 4.5 (concentrations are 2% by weight). d-e, CD spectra of P9G-Sup35 (d) and C8-Sup35 (e) in water (concentrations are 0.167% by weight), pH 4.5.
Figure S5-11. MALDI-ToF mass spectra of spermine-crosslinked MASP1 after crosslinking (left panel), 6 h after adding Tris buffer (middle panel), and 6 h after treating with MMP-2 in Tris buffer (right panel).

Table S5-1. Assignments of the observed peaks in MALDI-ToF mass spectra on the basis of a combination of MASP1 multimers with varying numbers of crosslinkers after the post-crosslinking treatment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak detected</th>
<th>Assigned Oligomer</th>
<th>Sample</th>
<th>Peak detected</th>
<th>Assigned Oligomer</th>
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</thead>
<tbody>
<tr>
<td>PCL1-crosslinked MASP1</td>
<td>5.83k</td>
<td>(1,2)</td>
<td>PCL2-crosslinked MASP1</td>
<td>5.87k</td>
<td>(1,2)</td>
</tr>
<tr>
<td></td>
<td>6.61k</td>
<td>(2,1)</td>
<td></td>
<td>6.62k</td>
<td>(2,1)</td>
</tr>
<tr>
<td></td>
<td>8.50k</td>
<td>(2,2)</td>
<td></td>
<td>8.38k</td>
<td>(2,2)</td>
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<td></td>
<td>10.12k</td>
<td>(2,3)</td>
<td></td>
<td>10.16k</td>
<td>(2,3)</td>
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<td></td>
<td>9.08k</td>
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<td></td>
<td>10.82k</td>
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<td></td>
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<td></td>
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<td>Spermine-crosslinked MASP1</td>
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</tr>
<tr>
<td></td>
<td>7.83k</td>
<td>(3,x)</td>
<td></td>
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<td>(3,x)</td>
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<td></td>
<td>10.36k</td>
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<td>10.30k</td>
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<td>15.42k</td>
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Figure S5-12. a-b. TEM micrographs of PCL1-crosslinked MASP1 filaments after 5 days (pH ~7). These TEM studies reveal that the crosslinked filaments (or networks) are stable at pH ~7. Solution pH was adjusted by adding NaOH.

Table S5-2. Assignments of the observed peaks in MALDI-ToF mass spectra on the basis of combinations of MAPS1 multimers and various degradation residues of the crosslinkers after 6 h incubation with MMP-2.

<table>
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<tr>
<th>Sample</th>
<th>Peak detected</th>
<th>Assigned Oligomer</th>
<th>Sample</th>
<th>Peak detected</th>
<th>Assigned Oligomer</th>
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<tr>
<td>PCL1-crosslinked MASP1</td>
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<td>PCL2-crosslinked MASP1</td>
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<td></td>
<td>5.63k</td>
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<td></td>
<td>11.51k</td>
<td>(3,3)</td>
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<td>weak signal</td>
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Supporting Information for Chapter 6

**Figure S6-2.** Molecular Characterizations of MASP2. A) Analytical HPLC chromatogram of MASP2. B) MALDI-ToF mass spectrum of MASP2.

**Figure S6-3.** Molecular Characterizations of MASP3. A) Analytical HPLC chromatogram of MASP3. B) MALDI-ToF mass spectrum of MASP3.

**Figure S6-4.** Emission spectra of Nile red in MASP2 (A), MASP3 (B), and MASP4 (C) solutions with various concentrations.
Figure S6-5. Time dependence of the mean squared displacement (MSD) of probing nanoparticles embedded in 4 mM MASP2, MASP3, and MASP4. The ensembles represent the average of MSD tracked particles. (mean ± SEM).
References


177. Ma, Q.G. & Wooley, K.L. The preparation of t-butyl acrylate, methyl acrylate, and styrene block copolymers by atom transfer radical polymerization: Precursors to amphiphilic and hydrophilic block copolymers and conversion to


Curriculum Vitae

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EDUCATION

<table>
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<tr>
<th>Institution</th>
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<th>Years</th>
<th>Advisor</th>
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<tr>
<td>Johns Hopkins University (JHU)</td>
<td>PhD, Chemical &amp; Biomolecular Engineering</td>
<td>09/2010–08/2014</td>
<td>Honggang Cui, PhD</td>
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<tr>
<td>National Taiwan University (NTU)</td>
<td>BS, Chemical Engineering</td>
<td>09/2005–06/2009</td>
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</table>

PROFESSIONAL EXPERIENCE

Graduate research assistant, Cui Lab, Dept. ChemBE, JHU 2010–current

- Employed chemical and bioconjugation strategies to design, synthesize, and purify anticancer drug-peptide conjugates (normally small molecules) to construct well-defined nanostructures for drug delivery. Utilized combinative techniques including MALDI-ToF mass spectrometry (MS), ESI MS, NMR, prep-HPLC, flash chromatography to identify or purify the molecules. Particularly interested in developing self-assembled nanotubes, and studying the stability, solubility, drug release kinetics, and the supramolecular morphology.

- Designed novel types of amphiphilic peptides to gain in-depth understanding of the connections between the design parameters of building blocks and their self-assembled structures. Utilized cryogenic transmission microscopy (cryo-TEM) and topological analysis in TEM to identify the supramolecular structures and monitor the kinetic process of self-assembly in aqueous environments.

- Designed amphiphilic peptide derivatives to fabricate functional hydrogels via the bottom-up approach to control the enzymatic degradation or rheological behaviors.

- Possessed 3+ years experience in the management and maintenance of core laboratory instruments including HPLC, peptide synthesizer, UV-Vis spectrometer and lyophilizer.

- Collaborated with MacKay lab (Pharmaceutical Science, University of Southern California) to investigate the assembled morphology of self-assembling protein polymers under cryo-TEM.

- Collaborated with Mao lab (Materials Sciences & Engineering, JHU) to design peptide-copolymer to develop enzymatically degradable polymers.

Teaching assistant, Dept. ChemBE, JHU 2011–2013

- Instructed weekly recitation sessions to undergraduate level Transport Phenomena (EN.540.304, Fall 2011 & 2012) to solve problems in fluid mechanics, heat transfer and mass transfer.

- Provided lectures in the class “Current Topics in Functional Molecular Assembly” (EN.540.407, Spring 2013) to introduce current advances of self-assembled materials in tissue engineering and regenerative medicine.
Undergraduate research assistant, Hsieh Lab, Dept. ChemE, NTU 2008-2009

- Participated in the development of porous scaffolds by polysaccharide composites. Evaluated the roles of composite formulation and freeze-gelation processing on mechanical strength, thermal behaviors, and microarchitecture. Equipped with techniques and knowledge in tensometer, differential scanning calorimetry (DSC), and scanning electron microscopy (SEM).

**TECHNICAL SKILLS**

- **Synthetic & analytical chemistry**: solid-phase peptide synthesis, organic synthesis, flash chromatography, HPLC, MALDI-ToF MS, ESI MS, NMR spectroscopy, UV-Vis, circular dichroism, fluorescent spectroscopy

- **Material characterizations**: TEM, cryo-TEM, SEM, dynamic light scattering (DLS), X-ray scattering, FTIR, DSC, tensometer

**HONORS AND AWARDS**

- Cancer Nanotechnology Training Center Fellowship, 2010–2012 (JHU)
- President Award (GPA rank within top 5%), Spring 2008, Fall 2009, and Spring 2009 (NTU)

**PUBLICATIONS**

In preparation


Submitted/In Revision


Published/Accepted


Selected Presentations


2. Self-assembly of ABC miktoarm star peptides and kinetic evolution of the supramolecular morphology. APS March Meeting, Baltimore, MD, 03/2013, poster presentation

3. Incorporating MMP degradable features into supramolecular filament hydrogels. 2012 AIChE Annual Meeting, Pittsburgh, PA, 10/2012, oral presentation

5. Tuning supramolecular filament networks assembled by amphiphilic mikto-arm star peptides: an approach from the molecular design. **86th ACS Colloids and Surface Symposium**, Baltimore, MD, 06/2012, *oral presentation*

6. ABC mikto-arm star peptides: building blocks and tuning units of supramolecular filaments and networks. **Gordon Research Conference (Colloidal, Macromolecular & Polyelectrolyte Solutions)**, Ventura, CA, 02/2012, *poster presentation*