Establishing Design and Assembly Rules for Peptide Oligonucleotide Chimeras (POCs)

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Submitted to the Graduate Faculty of the

Kenneth P. Dietrich School of Arts and Science in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2019

UNIVERSITY OF PITTSBURGH

DIETRICH SCHOOL OF ARTS AND SCIENCES

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University of Pittsburgh, 2019

Peptide Oligonucleotide Chimeras (POCs) are an emerging class of soft materials which offer tremendous potential in a variety of therapeutic applications owing to both the peptide and oligonucleotide components. These applications often depend upon the high selectivity, tunability and self-assembly behavior of the POCs. Therefore, a thorough understanding of the nature and self-assembling properties of POCs could allow for synthesis of POCs that meet the criteria of a specific application. The goal of this work is to probe the effects of hydrophobicity and β -sheet character on the self-assembly properties of POCs. Here, the nature of assembly and morphological changes of oligonucleotide-biphenyl-peptide conjugates (oligonucleotide = AACAATTATACTCAGCAA, AACAATTATACTCACCAA; peptide AAAYSSGAPPMPPF, AAAYVVAAPPMPPF, AAAYVFAAPPMPPF, = AA(AIB)Y(AIB)SG(AIB)PPMPPF) were studied over a range of salt concentrations (CaCl₂). The presence or absence of assembled structures, morphology of discrete nanostructures (spheres or fibers) and salt concentration at point of transition from spheres to fibers is probed for each analogue with respect to hydrophobicity and β -sheet character of the peptide. It was observed by transmission electron microscopy (TEM) that no ordered structures are formed in the absence of β -sheet character and higher hydrophobicity of peptides led to a shift from exclusively spheres to a combination of fibers and spheres at the same salt concentration. This study will provide the basis for the design of future POCs with programmable structures and properties.

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1.0 Introduction

1.1 Peptide Assembly

Certain peptides by virtue of their design can undergo spontaneous assembly into ordered nanoscale structures. One of the first reports of such self-assembling peptides AEAEAKA-KAEAEAKAK (EAK16) was made by Zhang et. al¹ in 1993. The well-defined sequences of such peptides allow them to undergo ordered self-assembly, resembling in part some mechanisms found in well-studied polymer assemblies. Following this report several designer peptides were synthesized that attracted interest for their potential applications in the fields of biomedical nanotechnology, tissue cell culture and molecular electronics. One common structural motif that has been repeatedly used to guide self-assembly and construct nanomaterials is diphenylalanine (L-Phe-L-Phe). This peptide is known to be associated with the pathogenesis of Alzheimer's disease^{2–4}. A variety of nanostructures have been reported from FF-based building blocks namely nanotubes, spherical vesicles, nanofibrils, nanowires, and ordered molecular chains. Subsequently, these structures have been used for bioimaging, biosensors, guest encapsulation, nanofabrication as well as drug delivery⁵ (Figure 1).



Figure 1. Various structures formed from FF-based building blocks and their applications.⁵

A key ingredient in the success of peptides as building blocks is their multitier structure (Figure 2). There are three hierarchical levels for peptides based on structure and conformation. The fourth and final structure is called a protein and is essentially a combination of peptides. The first level is the sequence of amino acids in the peptide chain. Amino acids are monomeric building units that contain terminal amine and carboxyl groups. They also have a whole spectrum of side chain structures including thiols, alcohols, aromatic groups, etc. These groups participate in chemical interactions and facilitate molecular recognition. The second-tier structure is a result of hydrogen bond formation between the highly polar amine and carbonyl groups alternating on the peptide backbone. Secondary structures include α helices, β sheets, random loops, turns, and coils. Secondary structures will vary depending on the sequence, planarity, hydrogen-bonding, steric crowding, repulsion or attraction of charged groups, and hydrophobic or hydrophilic character of substituent amino acids in the peptide. A tertiary structure is formed when a single peptide backbone has one or more secondary structures⁶. The interactions and bonds between side chains within a sequence determine the tertiary structure of the peptide. When several peptides come together, a protein sub-unit or the quaternary structure is formed.



N-terminal, internal and C-terminal modifications are often carried out to make peptides useful for diverse applications. For N-terminal modifications, acetylation is used to increase stability by preventing N-terminal degradation⁸, biotin-labeling is common in immunoassays⁹, and dansyl attachments are used in fluorescence-based assays, for example. Internal modifications include sulfide cyclization to increase enzyme stability¹⁰ and isotope labeling¹¹ to study their chemical, physical and biological properties.

Finally, the supramolecular structures of peptides are also dynamic in that they can reassemble depending on their environment and changing non-covalent interactions. The modularity, functionality and responsiveness of peptide-based assemblies thus make peptide building blocks worthy candidates for bottom-up fabrication of complicated nanostructures.

1.2 Peptide Conjugate Assembly

Designing synthetic self-assembling peptides requires a 'bottom-up' instead of a 'topdown' approach. In the 'peptide lego' system of self-assembly, the peptide sequence is

designed with alternating polar and non-polar amino acid residues that promote β -sheet formation while in the 'lipid-like peptide' system the building blocks contain a distinct headgroup with a hydrophobic tail that promotes self-assembly¹². Peptide motifs with aliphatic tails are often called peptide conjugates. In aqueous conditions, hydrophobic collapse of the aliphatic tails induces assembly of molecules into supramolecular one-dimensional nanostructures¹³. These nano-assemblies are comparable to proteins in structural diversity and biological functionality. They can form filaments, 2D-sheets, spheres, networks, tubes and helices (Figure 3). The Stupp group at Northwestern University is one of the pioneers of peptide amphiphilic (PA) design and assembly. Their PAs usually contain three domains: hydrophobic tail, hydrogen bonding peptide sequence and charged hydrophilic peptide sequence¹⁴. The hydrophilic portion promotes solubility, the hydrogen bonding sequence has a high propensity for β -sheet formation and the hydrophobic part promotes assembly in aqueous environments. These amphiphilic molecules can assemble into cylinders, ribbons, twisted structures, or aggregates of more than one fiber¹⁵. Peptide sequence and the nature of the alkyl tail strongly influence the β -sheet character, morphology, surface chemistry, and potential bioactivity of the PA. Other factors that induce assembly are pH, concentration and the presence of divalent ions^{16,17}. A few of these techniques will be used in our studies to drive and regulate assembly.



Figure 3. Nanostructures formed from peptide conjugates.¹⁸

1.3 Oligonucleotide Assembly

In addition to peptides, oligonucleotides are widely found in biological systems. The sequence-specific binding properties of DNA based on the Watson-Crick base-pairing interactions have been exploited to direct the assembly of materials at the nanoscale. The two widely used approaches to make highly programmable molecules are (a) hybridization-based and (b) nanoparticle-templated¹⁹. In hybridization-based techniques, specially designed oligonucleotides pair together to form intricate architectures like tiles²⁰ and scaffolds²¹. DNA tiles formed by helical domains with crossover junctions have been used in atomic force microscopy (AFM) characterization as topographical markers, for protein and nanoparticle capture and organization on 2D lattices^{22,23} and also in nano-robotics^{24–27}. DNA Origami, which relies on folding a multi thousand-base circular single-stranded DNA using shorter helper oligonucleotides, has become a powerful tool for generating a variety of complex and

dynamic 2D²⁸ and 3D²⁹ structures (Figure 4). These structures have been used to pick up nanoscale cargo for plasmonic applications and to build meta-material architectures. On the other hand, for nanoparticle-templated methods, rigid inorganic nanoparticle cores serve as the basis of the core geometry¹⁹. The molecules are designed such that the ligands attach to the nanoparticle via a head-group moiety and the oligonucleotide tail extends into the solution controlling thereby controlling stability and reactivity³⁰. The spatial confinement of the DNA strands leads to sharper melting transitions³¹, enhanced binding constants³² and elevated thermal stability³³. Using short DNAs with sticky ends that match the strands on the inorganic core, families of different superlattice symmetries have been constructed³⁴ (Figure 4).



Figure 4. DNA hybridization based origami (A) 2D patterns²⁸ (B) 3D structures²⁹. Nano-template based DNA assembly (C) Different crystal structures constructed from nano-particle building blocks of the same size and composition by changing the nature of the nucleic acid bonds: FCC lattice (Top), BCC lattice (Middle), AIB₂ type lattice (Bottom).³⁴

1.4 Characterization of Self-assembled Structures

Transmission Electron Microscopy (TEM). Analysis of peptide nanostructures with high resolution (0.2 to 0.5 nm for conventional TEM and 1 Å for HR-TEM) at a scale of a few nanometers or lower can be accomplished by TEM. This technique involves passing an electron beam through the specimen to form an image. Images are formed from the interaction of the electrons in the beam with the sample. The most common method of sample preparation involves applying the diluted solution onto the copper TEM grid and either letting it dry or wicking away the solvent. In case salt is being used, additional washes with water might be necessary. This is followed by either positive (uranyl acetate)³⁵ or negative (phosphotungstic acid)³⁶ staining. Another method of sample preparation is by embedding nano-fiber gels into epoxy resin, sectioning the prepared sample with microtome and then visualizing with TEM¹³. Cryo-TEM is also used to image assembled structures¹⁷. This involves snap freezing in liquid ethane to preserve morphology and avoid the drying effect. EF-TEM is an improved electron microscopy technique which filters electrons in a specimen according to energy besides scatter angle. This allows for greater contrast without staining³⁷.

Atomic Force Microscopy (AFM). AFM involves scanning the surface with a tip attached to a cantilever whose deflections are recorded and then used to generate an image. Usually, for peptide-based nanostructures, silicon wafers or freshly cleaved and cleaned mica surface is used as a substrate. The sample is drop-cast on the substrate and non-contact tapping mode is used to probe the structures. Zhou et al. visualized Fmoc-based peptide hydrogels by diluting and dropping onto mica surface³⁸. AFM imaging provides topological information for different nanostructures.

For DNA based superstructures, a variety of different AFM scanning modes can be used to provide more information about the structures. For example, phase contrast images provide information about mechanical properties of the sample³⁵. Height differences below 1 nm can easily be detected, depending on tip size and quality. Furthermore, sub-structural features of origami assemblies can also be visualized³⁵.

Circular Dichroism Spectroscopy (CD). CD is widely used to determine the secondary structure of proteins and peptides. α -helix, β -sheet, and random coil structures each give rise to a characteristic signal in the far-UV region (180 to 250 nm) of the CD spectrum. α -helices have a positive peak at 196 nm and negative peaks at 209 nm and 223 nm. β -sheets peak around 202 nm and have a characteristic dip at 219 nm. Negative peaks around 210 nm usually indicate polyproline (PPII) structure.

Fourier-transform Infrared Resonance Spectroscopy (FTIR). FTIR is used to determine the secondary structure of peptides from the strong amide I band. The amide I band position for the α -helical conformation is located at 1638 cm⁻¹. The β -sheet band frequency is located at a wavelength of 1610 cm⁻¹ and a weaker band associated with high-frequency vibration of antiparallel β -sheet structure is seen at 1680 cm^{-1 8}.

Optical Microscopy Techniques. Polarizing, fluorescence, and confocal microscopy, despite their lower resolution and magnification, provide a large amount of information about peptide nanostructures. In polarizing microscopy, polarized light interacts with the sample and generates a contrast between the anisotropic parts and the rest of the sample. Birefringency, a property of anisotropic domains of an assembly, helped Hatgerlink et al. show orientation of liquid crystalline phase of peptide amphiphile gels¹⁶. Another method involves staining the

peptide structures with dyes such as Congo Red that bind to β -sheet domains. Due to birefringence, these areas show up as yellow-green and are used to detect amyloid structures³⁹. Fluorescent dyes such as Alexa and Thioflavin T are also used to stain nanostructures, which are then observed by fluorescence and confocal microscopy. The FRET (fluorescence resonance energy transfer) method can be used for real-time imaging of dynamic processes⁴⁰.

1.5 Peptide Oligonucleotide Conjugates

In the late 1970s, the observation that synthetic oligonucleotides could modulate gene function^{41,42} through antisense technology captured the imagination of the scientific community and gave rise to tremendous advancement in methods for oligonucleotide synthesis. Simultaneously, synthetic peptides were emerging due to the solid phase peptide synthesis protocol (SPPS) developed by Robert Bruce Merrifield⁴³. Solid phase methods for manufacturing two of the most commonly found building blocks in biological systems–synthetic oligonucleotides from phosphoramidite monomers and synthetic peptides from protected amino acids–set the stage for the design of novel materials with interesting structural and functional properties.

Nature is replete with examples of supramolecular association between oligonucleotides and polypeptides (ribosomes, chromatin etc.) but instances of covalent conjugates between the two species are few and far between. In the rare cases that they are found, they are confined to specific biological systems and are difficult to adapt synthetically⁴⁴. For a long time, the research in this field has been limited to developing new methods to build these molecules⁴⁵. The two prominent methods of peptide oligonucleotide conjugate synthesis

are: (1) on-resin synthesis of entire peptide oligonucleotide conjugate molecule, and (2) separate synthesis and purification of peptide and oligonucleotide followed by coupling in solution (Figure 5).



Figure 5. Chemical approaches for peptide oligonucleotide synthesis.⁴⁶

Despite fewer steps in the first process, the second approach is preferred because the conditions required for the solid phase synthesis of peptides and oligonucleotides are incompatible with one another. For example, the cleavage of peptide from resin is accomplished under highly acidic conditions (95% trifluoroaceticacid) that will depurinate oligonucleotides. To circumvent this issue, one could use peptide nucleic acids (PNA),⁴⁷ but PNAs are very expensive compared to phosphoramidites and may introduce unwanted flexibility for certain applications. Additionally, the lack of charge on the PNA backbone creates solubility issues. For the second method, peptides and oligonucleotides are synthesized separately and linked together with or without a homo or hetero bi-functional linker. The common methods of purification are chromatography (e.g. reverse phase, anion exchange) and precipitation with ethanol, for example. Since oligonucleotides do not generally contain

reactive moieties that could be used for conjugation reactions, specially modified nucleobases are employed. These bases contain amines, thiols, azides, alkynes, iodides, alkoxyamines and aldehydes⁴⁶. For peptides, amines (lysine), carboxylic acids (glutamic acid, aspartic acid), thiols (cysteines) are often used for conjugation. If two or more of these group are present, non-canonical amino acids (NCAA) containing side chains such as azides and alkynes could be used to ensure specificity. Recently, oxidative coupling has also been used to synthesize peptide oligonucleotide conjugates⁴⁸.

Peptide oligonucleotide hybrid materials opened a whole new range of possibilities. The sequence-specific base-pairing interactions of nucleic acids would allow for complex architectures like DNA Origami^{19,28,29,49–55} and spherical nucleic acid based assemblies^{19,56–58}. Similarly, the rich diversity of amino acids would endow these hybrid biomolecules with highly tunable assembly and substrate recognition capabilities^{59–69}. Other useful properties of these conjugates was sensitivity to ionic strength, temperature induced size variation⁷⁰, pH change triggered release of an encapsulated compound⁷¹ and biological stimuli-promoted morphological transitions⁷². In the 1990s, cellular uptake of synthetic oligonucleotides was one of the final barriers to widespread adoption of antisense technology. The development of peptide oligonucleotide conjugates showed promise at overcoming this barrier. It was shown that oligonucleotides conjugated to peptides like CPPs^{73–75}, arginine-rich MTS⁷⁶, TAT⁷⁷ and some histidine-rich peptides⁷⁸ enhanced cellular permeability in cultured cells. Various aspects of peptide oligonucleotide conjugates were therefore studied, including their synthesis^{79,80}, cellular uptake⁸¹, and use as fluorescent probes, PCR (polymerase chain reaction) primers, and molecular tags⁸².

However, peptide oligonucleotide conjugates are often overlooked as programmable building blocks for the construction of soft materials, and only a handful of such studies which probe their assembly have been conducted in the last 10 years^{71,83–86}. Abraham et. al. showed that spherical structures synthesized from peptide-DNA hybrid materials under ambient conditions and low concentrations aggregate to form fibers at higher temperatures and concentrations⁸³. Gour et. al. demonstrated that nucleotide conjugation to the dipeptide FF results in a shift from fibrous to spherical morphology in the resulting structures. Further, they showed that encapsulated dye could be released from the structures upon a decrease in pH from 6.5 to 4.5, indicating that such materials could be useful in drug delivery applications. They then characterized the π - π and hydrogen bonding interactions using fluorescence spectroscopy^{71,84}. Zhang et. al. synthesized a micelle-forming brush-block copolymer using polycaprolactone. The material allowed for high surface density of nucleic acids. They also demonstrated target gene knockdown in vitro using this biodegradable material⁸⁷. Wengel and Jensen et al. simultaneously exploited the Watson-Crick base pairing of oligonucleotides and coiled coil protein domain formation of peptides to create protein like structures⁸⁵. Lim et al. took advantage of the hybridization of DNA, β-sheet formation of peptides and control of molecular degrees of freedom to create complex and controllable aggregates⁸⁶.

Efforts aimed at studying the nature and self-assembly of DNA-peptide conjugates, though novel, are few and far between. Studies that systematically examine the molecular factors that influence the assembly of DNA-peptide conjugates are required in order to further the field. Our vision is to create a class of highly modular and programmable peptide oligonucleotide conjugates comprising a peptide unit and an oligonucleotide unit bridged together by an organic linker. Each unit – peptide, oligonucleotide, and organic linker of the conjugate – can be individually modified, and the effect of changes introduced can be

independently studied. We call these modular tri-unit molecules peptide-oligonucleotide chimeras (POCs), and they are the subject of the research presented herein.

2.0 Effect of Hydrophobicity and Secondary Structure of Peptide on the Self-assembly Properties of Peptide Oligonucleotide Chimeras (POCs)

2.1 Peptide Oligonucleotide Chimera (POC)

Connecting peptides and oligonucleotides together via an organic linker yields a new class of molecule which we term peptide oligonucleotide chimera (POC). The peptide and oligonucleotide are attached to an organic core by CuAAC (copper catalyzed alkyl-azide coupling) 'click' chemistry (Figure 6). The Rosi group has demonstrated that these crosslinked biomolecules can assemble into new materials that have unique features and programmable assembly properties⁸⁸.



Figure 6. Anatomy of the POC structure: a doubly bio-conjugated entity, where a single stranded DNA sequence and a peptide sequence are connected to either side of an organic core.

Salt-based assembly studies showed that POCs formed morphologically precise structures: spheres or fibers depending on salt concentration and oligonucleotide length⁸⁸. Two distinct POCs were synthesized: $PO_{18}C$ with an 18-mer oligonucleotide and PO_6C having a shorter 6mer chain. The use of salt was crucial because in the absence of charge-shielding

cations, POCs resisted assembling in aqueous media due to their negatively charged oligonucleotide backbones.



Figure 7. (a) The charge ratio value is the ratio of positive to negative charges of the assembly solution (N = number of oligonucleotide bases; the '+2' results from the azido-functionalized T residue and the deprotonated COO⁻ terminus of the peptide). (b) The charge ratio of the assembly solution as a function of CaCl₂ concentration and oligonucleotide length. TEM images of PO₁₈C vesicles (c) before and (d) after concentrated CaCl₂ addition.

[POC] screenings revealed that assemblies were readily formed at 500 μ M. Solutions of 500 μ M POC were prepared in aqueous CaCl₂ solutions (10, 50, 150, and 300 mM). These solutions were heated to 80 °C to denature any non-specific aggregation states and then cooled to room temperature to arrive at preferred assembled structures. Transmission electron microscopy (TEM) was used to image the resulting assemblies. For PO₁₈C, defined circular structures were observed in 50 mM CaCl₂; fibers were exclusively observed in 300 mM CaCl₂ At 150 mM CaCl₂, a mixture of products was observed including circular/pseudo circular structures and fibers. Few ill-defined assemblies were observed at 10 mM CaCl₂. For PO₆C, fibers and spheres were observed at 10 mM CaCl₂, while at higher concentrations mostly twisted fibers and fiber conjugates were formed. This experiment demonstrated that by varying the charge ratio (Figure 7) the morphology of assemblies could be controlled. Decreasing the concentration of negative charge by shortening the oligonucleotide length and holding the calcium ion concentration constant caused an increase in charge ratio and a subsequent shift from spheres to fibers, while increasing the positive charge by increasing the salt concentration and holding the oligonucleotide length constant caused a decrease in charge ratio and a subsequent shift from fibers to spheres. These results confirm the hypothesis that altering the charge ratio should induce a morphological change, which can serve as a design rule for future exploration of POC-based assembly.⁸⁸

With this design rule in place, the next logical step was to investigate how self-assembly might be affected at different charge ratios due to slight modifications of the constituent components. Potential modifications include i) changes in oligonucleotide component: length, sequence or structure; ii) alterations to the organic linker component: aromatic/aliphatic nature, length, connectivity; and iii) changes to the peptide component: length, sequence, charge, secondary structure, hydrophobicity/hydrophilicity. This exercise will provide further insight into the nature of the self-assembly of POCs and also afford us a comprehensive set of design rules for this new family of soft materials.

2.1.1 Hypothesis and Goals

In this study, we focus on the peptide segment of the POC. Two parameters of the peptide segment were selected for modification: hydrophobicity and β -sheet forming character. Hydrophobic amino acids tend to have a greater propensity for engaging in β -sheet assembly^{89–91}, so if we substitute the amino acids in the wild-type peptide with more hydrophobic residues, we will simultaneously be increasing the hydrophobicity their β -sheet formation propensity of the peptide. Our first goal was to synthesize four 'mutants' of the wild-type peptide. Three mutants would have greater hydrophobicity and hence greater β -sheet propensity while the fourth would be designed with β -sheet breakers to prevent β -sheet formation and it would have

comparable hydrophobicity as the wild-type peptide. C_{14} (alkyl chain) tails would then be appended to this family of peptides in order to probe their assembly characteristics using both microscopy and spectroscopy. The C_{14} tails are employed to promote aggregation and assembly of the peptides. Our second goal was to synthesize peptide oligonucleotide chimeras with the wild-type and mutant peptides and study their self-assembly properties at different salt concentrations to analyse the change in assembly behaviour as a function of [salt] and peptide sequence. We hypothesized that increasing the hydrophobicity of the peptide would accelerate the self-assembly process. This would be visually manifested by a transition from spheres to fibers at lower salt concentrations than previously observed. We also hypothesized that the absence of β -sheet character for the POC synthesized with the mutant peptide containing β sheet breakers could prevent assembly and hence the formation of discrete nano-structures.

2.1.2 General Methods and Instrumentation

All chemicals were purchased from either Aldrich or Fisher and used without further purification. N₃C₄H₈CO-AAAYSSGAPPMPPF N₃C₄H₈CO- $(N_3-A_2PEP_{Au}),$ AAAYVVAAPPMPPF, N₃C₄H₈CO-AAAYVFAAPPMPPF, N₃C₄H₈CO-AA(AIB)Y(AIB)SG(AIB)PPMPPF were synthesized by Solid Phase Peptide Synthesis (Merrifield synthesis). Peptide oligonucleotide chimeras (POCs) were purified using an Agilent 1200 Series reverse-phase high-pressure liquid chromatography (HPLC) instrument equipped with an Agilent Zorbax 300SB-C18 column. POCs were quantified based on their absorbance at 260 nm and using the total extinction coefficient of DNA (195, 100 M⁻¹cm⁻¹ PO₁₈C). Spectra were collected using an Agilent 8453 UV-Vis spectrometer equipped with deuterium and tungsten lamps. Transmission electron microscopy (TEM) samples were prepared by drop-casting 4 µL of solution onto a 3-mm-diameter copper grid coated with

formvar. After 4 min., the excess solution was wicked away and the grid was washed with nanopure (NP) H₂O (4 μ L) and wicked away immediately. TEM images were collected with a FEI Morgagni 268 (80 kV) equipped with an AMT side mount CCD camera system. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) data were collected using an Applied Biosystem Voyager System 6174 MALDI-TOF mass spectrometer (negative reflector mode; accelerating voltage: 20 kV) with 3-hydroxypicolinic acid (3-HPA) as the ionization matrix. NP water (H₂O, 18.2 MΩ) was obtained from a Barnstead DiamondTM water purification system. CD experiments were conducted on Olis DSM 17 CD spectrometer with a quartz cuvette (0.1 cm path length) at 25°C. ATR-FTIR spectroscopy was conducted on Thermo Nicolet iS50 FTIR with PM-IRRAS and VCD instrument. Peptide conjugates were dissolved in 0.01 M HEPES buffer to afford a 0.1 mM solution. After 1 day, the solution was dialyzed against NP water using d-tube dialyzers (Millipore catalog number: 71505–3). The solution was concentrated and was drop-cast onto the ATR-FTIR substrate before collecting spectra.

2.2 Assembly and Spectroscopic Studies of C₁₄ Peptide Conjugate Family

2.2.1 Preparation of C₁₄ Conjugates

As mentioned previously, four mutant peptide sequences with increasing hydrophobicity were synthesized for this study. However, our knowledge of how these peptides behave in aqueous solutions was still limited. There was a need to study the nature of these sequences to gather more insight into their self-assembling properties and secondary structure formations. An established way to promote self-assembly is to conjugate peptide segments to alkyl chains, effectively creating peptide amphiphiles. A combination of two driving forces should promote assembly: the hydrophobic interactions between the alkyl tails and hydrogen bonding between the amino acids on the peptide segment. With this rationale in mind, four peptide conjugates were synthesized with C_{14} alkyl chains appended to the N-terminus of the peptide sequences (Figure 8).



Figure 8. Family of C₁₄ peptide conjugates with mutated regions highlighted.

All four peptide conjugates were purified using high performance liquid chromatography (HPLC) and characterized using matrix assisted laser desorption/ ionization (MALDI) (Figure 9).



Figure 9. MALDI data for C₁₄ peptide conjugates.

2.2.2 Assembly Studies

Solutions of 0.1 mM C_{14} peptide conjugates were left to stand overnight in 0.01 M HEPES Buffer. HEPES has been shown to be a useful media for peptide assembly in our previous work⁸⁸ and hence was our choice of solvent for this experiment. Furthermore,

incubation experiments in water and acetonitrile had not promoted significant fiber formation. Transmission electron microscopy (TEM) was used to image the resulting structures. (Figure 10). TEM images revealed that C₁₄-AAAYSSGAPPMPPF, C₁₄-AAAYVVAAPPMPPF and C₁₄-AAAYVFAAPPMPPF formed fibers but C₁₄-AA(AIB)Y(AIB)SG(AIB)PPMPPF only formed small aggregates. This was in agreement with what we expected to observe since the β -sheet breakers in the C₁₄-AA(AIB)Y(AIB)SG(AIB)PPMPPF conjugate usually prevent the formation of hydrogen bonds and hence suppress fiber formation.



C₁₄-AA(AIB)Y(AIB)SG(AIB)PPMPPF



Small aggregates

Figure 10. TEM images of C₁₄ peptide conjugate assemblies.

2.2.3 Spectroscopic Studies

After preliminary visualization of the results of incubation via electron microscopy, a more thorough investigation was performed using circular dichroism (CD) and Fourier-transform infrared spectroscopy (FTIR) to probe the molecular structure of the assemblies. In circular dichroism (CD) studies, a negative peak at ~205 nm is indicative of polyproline II (PPII) conformation in solution and a negative peak at ~215 nm is indicative of β -sheet conformation. Our experiments reveal that C₁₄-AAAYSSGAPPMPPF and C₁₄-AAAYVVAPPMPPF exhibit pronounced β -sheet structure. C₁₄-AAAYVFAPPMPPF has a broad signal, so we speculate that besides having PPII conformation, it might also have some β -sheet character^{92,93,94}. C₁₄-AA(AIB)Y(AIB)SG(AIB)PPMPPF however, exhibits only PPII character and has no negative peak between 210 nm and 220 nm (Figure 11). FTIR studies for C₁₄-AAAYVVAPPMPPF, C₁₄-AAAYVFAPPMPPF showed amide I bands at 1630 cm⁻¹, which indicates the presence of β -sheet secondary structure⁹⁴. No such band was observed for C₁₄-AAAYSSGAPPMPPF or C₁₄-AA(AIB)Y(AIB)SG(AIB)PPMPPF. However, there are examples in literature^{92,95} that show that AYSSGAPPMPPF has β -sheet character (Figure 12).



Figure 11. Circular dichroism (CD) studies of C₁₄ conjugate assemblies.



Figure 12. Fourier transform infrared spectroscopy (FTIR) studies of C14 conjugate assemblies.

2.3 Assembly Studies on POC Family

2.3.1 Synthesis

The POCs corresponding to the four peptides were synthesized using a solution phase coupling of peptides and oligonucleotides approach⁴⁶ following a previously established procedure⁸⁸ (Figure 13). For attaching the azide modified peptide (N₃-Peptide) and azide modified oligonucleotide (N₃-DNA) to the doubly alkyl modified biphenyl (BP) copper catalyzed azide alkyne coupling based click reaction was employed. The 18-mer oligonucleotide was first clicked onto the biphenyl core and purified using HPLC. This biphenyl oligonucleotide conjugate was then clicked on to the different peptides, desalted using a NAP-5 column, and purified by HPLC.



Figure 13. Procedure for POC synthesis.

2.3.1.1 Preparation of Azido-modified Oligonucleotide Conjugate (O18-N3)

In a typical procedure, syntheses were carried out from the 3' direction using controlled pore glass (CPG) beads possessing 1 µmol of adenine (Glen Research, dA-CPG #20-2001-10, (1000 Å, 38 µmol/g)). The CPG beads were placed in a 1 µmol synthesis column and Ultramild 3'-phosphoramidites (Glen Research, Pac-dA-CE phosphoramidite #10-1601-05, Ac-dC-CE phosphoramidite #10-1015-C5, iPr-Pac-dG-CE #10-1621-05, dT-CE phosphoramidite #10-1030-C5) and 5'-Iodo-dT phosphoramidite phosphoramidite (Glen Research, #10-1931-90) were then added using the standard 1 µmol protocol on an Expedite 8909 synthesizer. Note, a mild Cap A Mix (Glen Research, 5% Phenoxyacetic anhydride in THF, #40-4212-52) was also used for synthesis due to the lability of the iodo moiety. At the end of the synthesis, the beads were dried overnight and kept in a tightly capped vial at ambient conditions. The terminal iodo groups were substituted for azides using an established procedure⁹⁶. The CPG beads were kept in the columns while a saturated mixture of sodium azide in anhydrous dimethylformamide (DMF) was prepared (approximately 30 mg per 1 mL, per 1 µmol). Upon pulling up 1 mL of the mixture in a syringe, the column was firmly attached with an empty syringe on one end and the one containing the mixture in the other. The mixture was slowly passed over the CPG beads several times before either being left at ambient conditions overnight or placed in a shaker at 60 °C for one hour. The beads were then washed thoroughly with DMF and acetone before drying with nitrogen. The solid phase coupling reactions with the organic core were performed using these dry CPG beads.

2.3.1.2 Attachment of Azido-modified Oligonucleotides to Diacetylene Biphenyl Core

Dry CPG beads containing azide-modified DNA were placed in an Eppendorf tube. The biphenyl core (200 mM in DMF), tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 18 mM in DMF), CuSO₄·5H₂O (18 mM in DMF), and L-ascorbic acid (100 mM in DMF) were also added. The reaction mixture was then blanketed with nitrogen before capping and shaken for 24 hours at 25°C in an Eppendorf® Thermomixer® R (Eppendorf, #022670107) at 1000 rpm. It is important that the CPG beads are constantly agitated while mixing and not sitting at the bottom of the tube. Once the reaction was complete, the CPG beads were filtered using a one-side fritted 1 µmol Expedite DNA synthesis column (Glen Research, #20-0021-01), then the beads were washed with DMF ($5 \times 1 \text{ mL}$) and acetone ($5 \times 1 \text{ mL}$) mL) and dried with nitrogen. The beads were then placed in 1 mL of AMA (1:1 ammonia:methylamine solution) (CAUTION: Only fresh AMA solutions that are not more than two weeks old and have been kept in the refrigerator below 0°C should be used) at 65°C for 15 minutes to cleave the conjugates from the solid supports. Thereafter, the ammonia and methyl amine were removed by passing a stream of nitrogen over the solution. To the remaining material was added ultrapure deionized H₂O (affording roughly 1 mL at the end), and the resulting solution was filtered through 0.45 µm nylon syringe filter (Acrodisc® 13 mm syringe filter #PN 4426T). The filtered solution was purified using reverse-phase HPLC eluting with a linear gradient of CH₃CN and 0.1 M TEAA (5/95 to 45/55 over 32 min).

2.3.1.3 Attachment of Azido-modified Peptide on to the Biphenyl Organic Core

The POCs were prepared using copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC)97,98 in which N3-Peptide was reacted with the azido-modified 18mer conjugate. The following stock solutions were prepared: A, 198.3 mM CuSO4 in NP H2O; B, 37.3 mM THPTA in NP H2O; C, 2 M urea in NP H2O; and D, 60.6 mM sodium ascorbate in NP H2O. LyophilizedN₃-Peptide (125 nmol) was dissolved in 70 µL of DMF and 50 µL of NP H₂O and the solution was transferred to a vial containing On-N₃ (100 nmol). To this vial was added a mixture of A and B (1.05 µL A mixed with 5.58 µL B), 1.5 µL of C, and 13.8 µL of D. The vial was sealed with parafilm wrap, wrapped in aluminium foil, and stirred for at least 5 hours at room temperature. DMF/NP H₂O (1:1) was added to bring the total volume to 500 µL. The resulting solution was desalted using a NAP-5 desalting column (GE Healthcare Life Sciences, #17-0853-02). The eluted solution was purified using reverse-phase HPLC eluting with a linear gradient of CH₃CN and 0.1 M TEAA (5/95 to 45/55 over 32 min).

The POCs thus formed were characterized using matrix assisted laser desorption/ionization (MALDI) (Figure 14).



Figure 14. MALDI data of peptide oligonucleotide chimera.

2.3.2 Assembly Studies

In a 250 μ L plastic vial, lyophilized POCs (20 nmol) were dissolved in CaCl₂ solutions to yield the desired concentration. The solutions were sonicated for 2 min. and centrifuged briefly. The vials were placed in a 1.5 mL centrifuge tube containing water that was pre-heated at 90°C in an Eppendorf® Thermomixer® R (Eppendorf, # 022670107), and the POC solutions were allowed to incubate for 15 min. at 90°C. After incubation, the temperature setting was lowered 1°C every 5 minutes until the temperature reached 25°C. After cooling to 25°C, the POC solutions were removed from the 1.5 mL centrifuge tube and allowed to sit overnight at room temperature. TEM samples were prepared after 15 to 20 hours.

It was our experience from previous studies with DNA-BP-AAAYSSGAPPMPPF that discrete nanostructures were only formed for 500 µM solutions of POC or higher. Hence, this concentraton was chosen as the starting point for our assembly studies. Solutions of 500 µM POCs were prepared in 50 mM, 100 mM, 150 mM, 200 mM, 250 mM and 300 mM CaCl₂ salt solutions. The vials were heated to 90°C and then allowed to gradually cool to room temperature. They were then left at room temperature overnight. The presence and nature of assembled structures were probed using TEM. In the case of DNA-BP-AAAYSSGAPPMPPF, spheres are formed at 50 mM CaCl₂ concentration. At 100 mM CaCl₂ concentration, similar spherical structures are seen. However, at 150 mM salt concentration there is a shift in morphology from spheres to fibers. At higher concentrations (200 mM, 250 mM, 300 mM) only fibers are observed. For DNA-BP-AAAYVVAPPMPPF, which has a more hydrophobic peptide segment that is also more prone to forming β -sheet secondary structures, spheres are observed only for the lowest salt concentration (50 mM CaCl₂). At all higher salt concentrations, only fibers are observed. Following the same trend DNA-BP-AAAYVFAPPMPPF forms fibers from 50 mM CaCl₂ and up. The nature of these fibers are very different from those observed in the two previous cases. Hence, when moving from DNA-BP-AAAYSSGAPPMPPF to DNA-BP-AAAYVFAPPMPPF there is a morphological shift from spheres to fibers at lower salt concentrations and predominantly fibers at higher ones (Figure 15). For DNA-BP-AA(AIB)Y(AIB)SG(AIB)PPMPPF, no discrete structures were observed at any salt concentration.



Figure 15. TEM images of POC assemblies at varying salt concentrations (Scale bar: 1 μ m) DNA-BP-AAAYSSGAPPMPPF at (a) 50 mM CaCl₂ (b) 100 mM CaCl₂ (c) 200 mM CaCl₂ (d) 300 mM CaCl₂; DNA-BP-AAAYVVAPPMPPF at (e) 50 mM CaCl₂ (f) 100 mM CaCl₂ (g) 200 mM CaCl₂ (h) 300 mM CaCl₂; DNA-BP-AAAYVFAPPMPPF at (i) 50 mM CaCl₂ (j) 100 mM CaCl₂ (k) 200 mM CaCl₂ (l) 300 mM CaCl₂.

2.4 Conclusion

Our results show that the hydrophobic interactions are integral to the formation of discrete nanostructures and that increasing peptide hydrophobicity initiates a shift from spherical to fibrous morphology. With an increase in salt concentration, there is a transition from vesicles to fibers in all three cases due to increased charge shielding. Holding the salt concentration constant, the higher the hydrophobicity of the peptide, the greater its tendency to form fibers. This is evident from the fact that from DNA-BP-AAAYSSGAPPMPPF to DNA-BP-AAAYVFAAPPMPPF in 50 mM CaCl₂, there is a transition from spheres to a combination of spheres and fibers. For DNA-BP-AAAYSSGAPPMPPF, the transition from spheres to fibers occurs between 100 mM and 150 mM, for DNA-BP-AAAYVFAAPPMPPF this occurs between 50 mM and 100 mM, while for DNA-BP-AAAYVFAAPPMPPF both spheres and

fibers are seen in 50 mM CaCl₂. It is also observed that DNA-BP-AA(AIB)Y(AIB)SG(AIB)PPMPPF, which has roughly the same hydrophobicity as AAAYSSGAPPMPPF but no β -sheet forming character, does not form assemblies in 50 mM or 300 mM CaCl₂.

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Supramolecular Assembly of Peptide Amphiphiles

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Acc. Chem. Res., 2017, 50 (10), pp 2440–2448 DOI: 10.1021/acs.accounts.7b00297 Publication Date (Web): September 6, 2017 Copyright © 2017 American Chemical Society



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