Functional Hybrid Biomaterials based on Peptide-Polymer Conjugates for Nanomedicine

By
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by

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Abstract

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The focus of this dissertation is the design, synthesis and characterization of hybrid functional biomaterials based on peptide-polymer conjugates for nanomedicine. Generating synthetic materials with properties comparable to or superior than those found in nature has been a “holy grail” for the materials community. Man-made materials are still rather simplistic when compared to the chemical and structural complexity of a cell. Peptide-polymer conjugates have the potential to combine the advantages of the biological and synthetic worlds- that is they can combine the precise chemical structure and diverse functionality of biomolecules with the stability and processibility of synthetic polymers. As a new family of soft matter, they may lead to materials with novel properties that have yet to be realized with either of the components alone.

In order for peptide-polymer conjugates to reach their full potential as useful materials, the structure and function of the peptide should be maintained upon polymer conjugation. The success in achieving desirable, functional assemblies relies on fundamentally understanding the interactions between each building block and delicately balancing and manipulating these interactions to achieve targeted assemblies without interfering with designed structures and functionalities. Such fundamental studies of peptide-polymer interactions were investigated as the nature of the polymer (hydrophilic vs. hydrophobic) and the site of its conjugation (end-conjugation vs. side-conjugation) were varied.

The fundamental knowledge gained was then applied to the design of amphiphiles that self-assemble to form stable functional micelles. The micelles exhibited exceptional monodispersity and long-term stability, which is atypical of self-assembled systems. Thus such
micelles based on amphiphilic peptide-polymer conjugates may meet many current demands in nanomedicine, in particular for drug delivery of hydrophobic anti-cancer therapeutics.

Lastly, biological evaluations were performed to investigate the potential of micelles as drug delivery vehicles. In vitro cell studies demonstrated that the micelles can be used as a delivery vehicle to tailor the cellular uptake, time release, and intracellular trafficking of drugs. In vivo biodistribution and pharmacokinetic experiments showed long blood circulation. This work demonstrates that peptide-polymer conjugates can be used as building blocks to generate hierarchical functional nanostructures with a wide range of applications, only one of which is drug delivery.
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# Chapter 1

**Opportunities of Peptide-Polymer Conjugates**

## 1.1. Introduction

## 1.2. Peptides/proteins

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1.2.2 Secondary structure

1.2.2.1 α-helix

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1.2.3 Tertiary structure

1.2.3.1 Coiled-coil

1.2.3.2 β-structures

1.2.4 Stability of protein folds

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## 1.3. Advantages of peptide-polymer conjugates

## 1.4. Perspective and outlook

## 1.5. General approach and synopsis of subsequent chapters

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§ 1.1 Introduction

The work described in this dissertation focuses on gaining a fundamental understanding of peptide-polymer conjugates, a new class of hybrid materials based on peptides and synthetic polymers, and applying the fundamental knowledge gained towards rationally designing functional nanomaterials for potential applications in nanomedicine. From a materials point of view, man-made materials are still rather simplistic when compared to the chemical and structural complexity of a cell. Generating synthetic materials with properties comparable to or superior than those found in nature has been a “holy grail” for the materials community. It remains a challenge to generate materials with hierarchical assemblies down to the atomic level that have precisely tailored chemical heterogeneities and external stimuli-responsive, as biomolecules inherently do. Peptide-polymer conjugates have the potential to combine the advantages of the biological and synthetic worlds- that is they can combine the precise chemical structure and diverse functionality of biomolecules with the stability and processibility of synthetic polymers. As a new family of soft matter, they may lead to materials with novel properties that have yet to be realized with either of the components alone.

Although assemblies of polymeric nanostructures can be readily produced over macroscopic distances\(^1\) and numerous monomers are readily available for immense modularity,\(^2\) precisely defining the chemical constitution, uniformity, and lengths of synthetic polymers remains elusive. Thus, achieving structural control at the molecular level has yet to be realized with synthetic polymers alone. In comparison, proteins have diverse chemical heterogeneities that govern intra- and inter-molecular interactions and underpin the folding of natural proteins into well-defined structures over multiple length scales. This also leads to functionality and stimuli-responsiveness. However, the drawback is that these protein-based materials are limited by their stability and degradation. One route to overcome some of the deficiencies of synthetic materials and biological materials alone is to form hybrid materials, such as peptide/protein-polymer conjugates, which integrate the structural richness of natural materials with the stability of synthetic materials.\(^3-6,7\)

This chapter will first introduce and highlight individual biological components available for the generation of hybrid materials. In this chapter and throughout the rest of this dissertation, a great emphasis will be placed on a fundamental understanding of the thermodynamics of self-assembly of this multicomponent system. This will lead to a discussion of the many possibilities afforded by these materials. Lastly, my perspective and outlook of this burgeoning field will conclude this chapter, which provides the basis and motivation for the work described in the remainder of this dissertation.
§ 1.2 Peptides/proteins as building blocks for hybrid materials

Peptides and proteins are linear polymers that are made up of monomers known as amino acids, whose chemical structure is shown in Figure 1.1a. Proteins are much larger in length than peptides and describe complete, biological macromolecules with a stable conformation, whereas peptides are usually short amino acid oligomers. There are 20 natural amino acids, which are tabulated and classified in Figure 1.1b based on the chemical nature of their side-chain. Each amino acid has a corresponding three letter and one letter code, which are listed in the table. An important characteristic of an amino acid side-chain is its polarity, which largely dictates protein folding. Nonpolar side-chains, such as methyl groups, are hydrophobic and prefer not to interact with water, whereas polar side-chains, such as carbonyl, amino, hydroxyl, and sulfhydryl groups, are hydrophilic and prefer to interact with water. The amino acids can join together into long chains by forming amide bonds between the -NH$_2$ group of one amino acid and the -COOH group of another. The sequence of amino acids, which constitutes the primary structure, ultimately determines the higher order secondary, tertiary, and quaternary structures of the protein, which are critical to its function. The terms “primary,” “secondary,” “tertiary,” and “quaternary” structure emphasize the hierarchical nature of protein structure, which makes them unique in comparison to synthetic polymers.

1.2.1. Primary structure

The primary structure refers to the sequence of the different amino acids in a peptide or protein. The two ends of the peptide chain are referred to as the carboxyl terminus (C-terminus) and the amino terminus (N-terminus), based on the nature of the free group on each extremity. The primary structure is typically read from the N-terminus to the C-terminus, using the three letter or one letter amino acid codes. The primary structure is unique to a particular protein and ultimately defines the structure and function of that protein.

1.2.2. Secondary structure

Secondary structure refers to the local, specific, geometrical shape of a peptide. The conformations of peptide backbones are restricted by steric clashes between backbone and side-chain atoms. The allowed values for the backbone dihedral angles (φ, ψ), shown in Figure 1.1c, define the spatial orientation of the peptide. This, coupled with the formation of hydrogen bonds between the –NH– and –CO– groups of the backbone, leads to regular secondary structures, such as the α-helix and the β-sheet, the two most predominant types of protein secondary structures. Turns and loops link the more regular
secondary structural elements, and random coils describe conformations without regular secondary structures.

Figure 1.1. (a) Chemical structure of amino acids. (b) Table listing the 20 natural amino acids categorized by the chemical nature of the side-chain R group. (c) Representation of the dihedral angles in a peptide backbone.

1.2.2.1. α-helix

The most common secondary structure found in natural proteins is the α-helix, shown in Figure 1.2a. The α-helix is a right-handed coil with backbone dihedral angles of $\phi = -60^\circ$ and $\psi = -45^\circ$, in which each amino acid residue corresponds to a $100^\circ$ turn in the helix and a rise of 1.5 Å along the helical axis, so that the helix has 3.6 residues per turn, and the backbone –CO– group of the $i^{th}$ residue forms a hydrogen bond with the –NH– group of the $i+4^{th}$ residue. Thus all –NH– and –CO– groups, except the N- and C-termini, are involved in hydrogen bonds. Helices observed in natural proteins can range from 4 to
over 40 residues in length. The radius of the helix, excluding side-chains, is 2.3 Å. The thermodynamically driven process of helix folding is a balance of the entropic cost associated with the folding or constraining of the peptide chains and the stabilizing effects of forming intra-hydrogen bonds. Because all of the backbone amide groups are involved in intra-chain hydrogen bonds, the interactions of helices with other peptides or molecules occur exclusively through side-chain interactions. Amphipathic helices, in which one face of the helix is polar and the other is nonpolar, are common, thus allowing for the formation of higher order structures via intermolecular interactions, such as hydrophobic and electrostatic interactions, which will be discussed later.

Figure 1.2. (a) Structure of an α-helix which shows hydrogen bonds between the –CO– group of the i\textsuperscript{th} residue and the –NH– group of the i+4\textsuperscript{th} residue. (b) Structures of parallel and anti-parallel β-sheets which show the difference in inter-peptide hydrogen bond patterns.

1.2.2.2. β-sheet

The other common secondary structure is the β-sheet, shown in Figure 1.2b. β-sheets consist of two or more β-strands connected laterally by backbone hydrogen bonds, generally forming a twisted, pleated sheet, with dihedral angles of φ = -130° and ψ = 120°. A β-strand is a stretch of peptide consisting of amino acids with a repeating hydrophobic (H), hydrophilic (P) residue pattern (HPHPHP...) with the backbone in an almost fully extended conformation. β-strands are arranged adjacent to other strands and form an extensive hydrogen bond network with their neighbors, in which the –NH– groups in the backbone of one strand form hydrogen bonds with the –CO– groups in the
backbone of adjacent strands. Therefore, unlike α-helices that form intra-hydrogen bonds, β-sheets form inter-hydrogen bonds. Because peptide chains are directional, with an N- and C- terminus, β-strands are also directional and can form sheets in a parallel, antiparallel or mixed arrangement, which are shown in Figure 2b. They form a pleated structure, with the side-chains of the peptide sequence pointing alternately above and below the plane of the β-sheet. With the alternating HP pattern, one face of the β-sheet contains hydrophobic residues while the other contains hydrophilic residues. Analogous to the case of amphipathic α-helices, this allows for the formation of higher order structures.

### 1.2.3. Tertiary structure

Proteins perform a diverse array of functions and recognize many different molecules in the cell by detailed three-dimensional interactions. These diverse three-dimensional protein structures that nature has evolved are termed tertiary structures, and are determined by the primary structure and the different possible ways to pack the regular secondary structural elements. It is rather impressive that nature has evolved to rely on the arrangement of the few common secondary structural elements to give rise to a large library of proteins that exist. The tertiary structure of a protein refers to the three-dimensional structure of the folded protein, and it is usually the simplest structural element capable of performing a function. Secondary structural elements pack against each other to form simple tertiary structural motifs, and several motifs usually combine to form compact folded protein structures, also known as quaternary structures. There exist many different tertiary structures; two well-known examples, based on α-helices and β-sheets, are coiled-coils and β-barrels. They are commonly found in proteins and are key to functions, such as enzymatic activity, transport, signaling, and, redox reactions.

#### 1.2.3.1. Coiled-coil

A common tertiary motif based on α-helices is the coiled-coil, as shown in Figure 1.3b. Coiled-coils are left-handed supercoils of multiple right-handed α-helices. Each helix is amphipathic, with a hydrophobic face and a hydrophilic face, and has a characteristic heptad repeat, labeled “abcdefg”, shown in Figure 1.3a. The a and d positions of the helical wheel are usually occupied with hydrophobic residues so that the side-chains from multiple peptides can pack together to form the hydrophobic core of the bundle, giving rise to much of the stability and specificity of coiled-coils. Residues in positions e and g border the hydrophobic core and are typically charged residues which form salt bridges between helices that determine the relative chain alignment and orientation, in addition to enhancing stability. Therefore, the periodicity of hydrophobic and hydrophilic residues of a coiled-coil peptide follows the pattern, HPPHPP.8,9
Figure 1.3. (a) Schematic drawing of the helical wheel of a coiled-coil 3-helix bundle, depicting the heptad “abcdefg” repeat of an α-helix. The a and d residues are hydrophobic and well packed in the interior of the helix bundle, colored green. (b) Crystal structure of a coiled-coil 3-helix bundle, known as 1coi in the Protein Databank. (c) Schematic drawing of a β-barrel.

1.2.3.2 β-structures

A common tertiary motif composed of β-sheets is the β-barrel. A β-barrel is a large beta sheet that twists and coils to form a closed structure in which the first strand is hydrogen bonded to the last, as depicted in Figure 1.3c. β-strands are typically anti-parallel, with hydrophobic residues oriented in the interior of the barrel to form a hydrophobic core and the polar residues oriented toward the exterior of the barrel. β-sheet structures are also commonly recognized and studied in their amyloid form. These amyloid structures are insoluble aggregates that are linked to a range of diseases, most notably Alzheimer's disease. They form from the stacking of β-strands that lie perpendicular to the axis of the fibril. β-strands are hydrogen bonded to form β-sheets that run parallel to the fibril axis. These stacks of β-sheets then bundle together to form the fibril. The diameter of most fibrils tends to be around 10 nm, with its length varying drastically. Because of the harmful effects linked to such amyloidoses, much research has been done in this area to gain fundamental understanding of the structure and the interactions governing their self-assembly, with the eventual goal of developing a cure for linked diseases. The increased knowledge of β-sheet structures has also led the materials scientist to use them as useful nanostructured materials. They are attractive because they form the distinct hierarchical levels of structure that span length scales from nanometers to microns, as β-strands assemble to form β-sheets, which further stack to ribbons, then fibrils, to fibers with a well-defined structure. Though many studies in the field of peptide-based materials has been based on β-sheet structures, they will not be discussed further here because there already exist many comprehensive references. Rather, the
work in this dissertation will focus solely on helix-based structures.

1.2.4. Stability of protein folds

The conformational stability of a protein is mainly governed by the amino acid sequence in a given environment. Local conditions, such as solvent, pH, and ionic strength affect the conformational properties of a protein. The role of solvent is of particular importance for protein structure stabilization, so water-soluble proteins in aqueous media and membrane proteins embedded in lipid bilayers require different consideration. The hydrophobic effect, which is the tendency of nonpolar molecules to aggregate in aqueous solution to exclude water molecules, is the major driving force in protein folding. Globular proteins are surrounded by water molecules, so a large driving force for folding is the packing of hydrophobic side-chains into the interior of the molecule, thus creating a hydrophobic core and a hydrophilic surface. The stabilization of protein structures in water represents a delicate balance between the conformational entropy of the polypeptide chain, which favors unfolding, and various stabilizing energetic contributions, such as hydrogen bonds, and hydrophobic, van der Waals, and electrostatic interactions. The energy scales for each of these contributions are small, on the order of kT, so fluctuations in the environment, such as pH, ionic strength, and temperature changes, may have a large impact. In order to pack hydrophobic side-chains in the interior of a protein, the backbone must also fold into the interior. The main chain is highly polar and therefore hydrophilic, so there is a great energetic penalty for removing it from the polar environment. This is alleviated by the formation of intrahydrogen bonds between the backbone NH and CO groups via the folding of secondary structures.

Membrane proteins, on the other hand, are embedded in the interior of a lipid bilayer, so the presence of hydrophilic residues on their exterior is energetically unfavorable. The hydrophobic interior of a lipid bilayer, like the hydrophobic core of a protein, provides no hydrogen bonding donors or acceptors. The energetic penalty for inserting a polar amino acid into the lipid bilayer can be very substantial, unless the hydrogen bonding capacity of the group is satisfied. Therefore, all membrane spanning segments form secondary structures, with polar side-chains rarely inserted into the lipid bilayer. The unique conformation of a native protein is determined by a large number and a large variety of non-covalent interactions, which severely restrict the possible conformational space that is available due to rotations around each single bond of the backbone. A thermodynamically stable conformation arises from the minimization of the overall free energy of interaction resulting from all the intra-molecular and inter-molecular contributions.
1.2.5. Peptides as building blocks for hybrid materials

Peptides are a desirable class of building blocks because they are able to self-assemble into the native structure that is encoded by their primary sequence. This well-defined structure leads to a diverse and complex array of functions. Therefore, the main advantages of peptides or proteins include hierarchical self-assembly, chemical functionality, selectivity and specificity, and dynamic response to external stimuli. The hierarchical structure provided by peptides allows for precise and well-defined building blocks that are identical from one to the next and are ordered over multiple length scales, from the molecular level to higher order tertiary structures. The chemical functionality possible with proteins is exemplified by the diverse functions that nature has evolved, as proteins are essential to organisms and participate in virtually every cellular process. A majority of these functions relies on the protein’s ability to bind other molecules specifically and with high affinity. This binding capability is mediated by the tertiary structure of a protein, which defines the binding pocket. The structures and interactions of biomolecules are largely dictated by non-covalent interactions. They are designed to undergo dynamic and reversible conformational transformations upon changes in pH, temperature, ionic strength. Each of these can be varied to tailor or control the structure of the protein, so that stimuli-responsiveness can be engineered, possibly leading to responsive materials. Because all of these characteristics of proteins are largely determined by structure, it is important to retain the natural structure of a protein, and thus its functionality, when attempting to incorporate it into hybrid materials.

In the case of synthetic polymers, they are usually composed of only a few types of monomers, which limits their chemical heterogeneity. Although polymers can form hierarchical structures, it is difficult to obtain atomic level structural control and built-in functionality at the molecular level. Figure 1.4 depicts the difference in hierarchical assembly between peptides and synthetic block copolymers (BCPs). While BCPs can form micellar aggregates with a simple core-shell fine structure, peptide organization leads to distinct nanostructures with precise hierarchical inner structures, from primary to quaternary structures. There exist many possibilities available for the biomolecular block, as exemplified by the various protein structures found in nature. To provide a concise survey of the field, the remainder of this chapter will focus largely on the use of helix based-peptides as building blocks for hybrid materials, with an emphasis on coiled-coil helix bundles as a promising option.

1.2.6. Promise of coiled-coils

Engineering minimalist protein structures that self-assemble and reproduce native-like function, thus simplifying the system from a complex, globular protein to a relatively short, precisely folded peptide, is a valuable strategy for incorporating biomolecular building blocks to generate useful materials. The coiled-coil is one of the simplest
tertiary-structural motifs and is the foundation of many of the functionalities of natural proteins, such as enzymatic activity, signal transfer and redox chemistry.\textsuperscript{17,18} Relatively short peptides can self-assemble into a coiled-coil to form a simplified version of a globular protein that is more robust and better-defined in structure, while still being able to perform the specific function of the native protein.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{peptide_protein_polymer.png}
\caption{Schematic illustration of the hierarchical self-assembly of peptides (\textit{secondary structure}: locally defined sub-structures in a single protein molecule; \textit{tertiary structure}: spatial arrangement of the secondary structures in a 3D structure of a single protein molecule; and \textit{quaternary structure}: arrangement of tertiary structure subunit assembly). In contrast, the limited hierarchal organization of synthetic block copolymers is shown on the right, with the formation of simple spherical micelles, cylindrical micelles, or vesicles.}
\end{figure}

Peptide-polymer conjugates based on coiled-coil peptides present a versatile strategy for generating hybrid functional materials.\textsuperscript{19-21} BCPs containing leucine zippers, a common coiled-coil, have been used to prepare hydrogels where the formed helix bundle acts as a physical crosslink.\textsuperscript{19-21} Coiled-coils have also been used as carriers for drug and gene delivery.\textsuperscript{22,23} As a structural motif, though, the functionality of coiled-coils go far beyond what has been explored to date, so there exists a lucrative area for further
study. For example, they should be able to perform highly-selective transport and enzymatic reactions and transduce signals to activate downstream reactions.\textsuperscript{24-26} The selectivity, sensitivity and responsiveness afforded by coiled-coils are far superior to those possible with synthetic materials alone, and should thus be exploited.

\textit{De novo} protein design (design with novel amino acid sequences) provides tremendous versatility and tailorability in the use of coiled-coils as building blocks.\textsuperscript{8,17,27,28} By design, the interior of the helical bundle can be engineered to obtain specific binding affinities to various natural and unnatural prosthetic groups or to form channels of different shapes and sizes.\textsuperscript{17,29,30} While the interior of the helix bundle performs sophisticated reactions through specific recognition and binding, the exterior of the helix bundle influences the interaction with the environment.\textsuperscript{27,31-33} \textit{De novo} protein design has shown that the exterior of the helix bundle can be tailored with specific patterns of charge or hydrophobicity, so as to facilitate insertion into vesicles, orientation at interfaces, or assembly on solid substrates, while preserving the interior of the helix bundle, i.e. its designed functionality.\textsuperscript{30,34-37} Since the interior can be tailored independently from the exterior, a sequence may be designed so that a peptide with a desired structure and functionality can be incorporated into the environment of choice.\textsuperscript{27} For example, the amino acids on the exterior of the transmembrane region of a natural protein have been mutated to modify the protein from being lipid-soluble to water-soluble without interfering with its built-in functionality.\textsuperscript{38} To mimic the function of natural proteins, such as signaling or enzymatic reactions, the bundle interior can be designed to bind specific prosthetic groups with high selectivity and precision in their spatial arrangement, and the dissociation constants can be tailored from the sub-nanomolar to the micromolar.\textsuperscript{32,38-43} A large library of coiled-coils are readily available, capable of transporting protons or positioning chromophores for light induced charge separation.\textsuperscript{38,44-47} Helix bundles can also be de novo designed to incorporate stimuli responsiveness.\textsuperscript{48} \textit{De novo} peptides have both chemical and structural diversities that are comparable to or greater than those found in natural materials, thereby significantly expanding the repertoire of these building blocks by enabling their design to meet specific needs. These recent developments in de novo protein design clearly demonstrate the possibility to custom design building blocks with novel properties that are not possible with synthetic materials alone.

\textit{De novo} designed peptides, in general, are much more robust in comparison to their natural counterparts. Folding for a short peptide sequence is a much simpler process compared to that for large natural proteins.\textsuperscript{49} For most \textit{de novo} designed peptides, folding is fully reversible,\textsuperscript{50} and they tend to have better stability and can maintain their structure against changes in temperature and solvents.\textsuperscript{51} Thus, they can retain their designer functionalities under conditions that are unsuitable for their natural counterparts.\textsuperscript{38} However, they are still subject to proteolysis and degradation, and it remains a challenge to process them into functional materials over macroscopic distances.\textsuperscript{51,52} To take full advantage of the unique properties offered by coiled-coils, controlling their macroscopic assembly is essential. For example, helix bundles must be oriented normal to a membrane...
or substrate interface in order to mimic natural transmembrane proteins. Various strategies include designing distinctive charge patterns and hydrophilic/hydrophobic patterns along the exterior of a coiled-coil so that they can be readily inserted into vesicles or macroscopically oriented at polar/non-polar interfaces.37,53-54

§ 1.3 Advantages of peptide-polymer conjugates

Peptide-polymer conjugates have the potential to synergistically combine the advantages of their individual constituents, while minimizing their respective liabilities.3,7,19,20,55-60 As discussed above, peptides provide the precise chemical, structural, and functional specificity that synthetic polymers lack, while synthetic polymers provide biomolecules with improved processibility, solubility, and stability against temperature, pH, organic solvents, and degradation.61,62 Therefore, the synthetic polymers could act to protect, deliver and template the peptides at the nanometer scale, while the peptide bundles could provide molecular level control over the chemical heterogeneity to trigger, direct and execute built-in functionalities.55,63 Together, they form a system that is capable of responsive hierarchical self-organization over three length scales. On the smallest length scale, peptide sequences direct folding into regular secondary structures, which are dictated mainly by hydrogen bonds. They are then capable of adopting tertiary structures, which rely on a combination of hydrophobic interactions, hydrogen bonds, and electrostatic interactions for stability and specificity. Lastly, microphase separation between the peptide and synthetic polymer offers organization at the highest length scale. This is analogous to the well-studied phase separation between traditional synthetic diblock copolymers, which is driven by the incompatibility between the two blocks and the covalent bond that constrains them together.

The phase-separated morphologies available to diblock copolymers can be tuned by tailoring the molecular weight and composition of each block and the solvents used in processing. The self-assembly of polymeric materials in the bulk and in thin films can be readily obtained over arbitrary distances. The current state of knowledge uses synthetic polymers alone to achieve order over multiple length-scales. When two or more polymers are connected together to form block copolymers, phase separation into tens of nanometer features occurs. Within the microdomain, polymer crystallization can add a further layer of order. Although crystal structures of polymers are not available at high resolution, as with natural proteins, it is still possible to get sub-10 nm control.

Peptide-polymer conjugates can take advantage of existing processing techniques and strategies to direct polymeric nanostructures. In fact, it should be possible to assemble helix bundles into ordered arrays to obtain macroscopic responses similar to those seen with natural proteins. Various functions, both natural and non-natural, can be incorporated into the peptide block through sequence design and modification, and the great monomer selection available for the synthetic block provides the ability to mediate
the interactions between the peptide and its environment and to control the self-assembled structure in a predetermined way. Furthermore, the peptide’s responsiveness to external stimuli can be exploited to construct ‘smart’ materials that undergo a change in size or structure, e.g. helix to coil transition, when desired. Therefore, the realization of peptide-polymer conjugates as novel building blocks for both biological and non-biological applications is clearly possible.

However, for peptide-polymer conjugates to reach their full potential as useful materials, the structure and function of the peptide should be maintained upon polymer conjugation in both the solution and the solid state. The success in achieving desirable, functional assemblies relies on fundamentally understanding the interactions between each building block and delicately balancing and manipulating these interactions to achieve targeted assemblies without interfering with designed structures and functionalities.

§ 1.4 Perspective and outlook

The vast motivation behind all the work in the field of peptide-polymer conjugates can be divided into three categories: fundamental science, self-assembly, and applications (both biomedical and non-biological). As depicted in Figure 1.5, these divisions are not mutually exclusive and naturally contain some overlap, as knowledge gained in one sector may be prerequisite or applicable to another. Further work in this field will rely on a constant balance between these three motivating factors, as substantial progress in applications development may not be possible without the others.

The first and perhaps most important area is establishing a fundamental understanding of the interactions involved in peptide-polymer conjugates. As more sophisticated peptide sequences are explored, more factors enter into the self-assembly of the system. Not only do peptide-polymer enthalpic interactions have to be considered, but also do polymer-polymer interactions and peptide intra- and inter-molecular interactions. Various non-covalent interactions, such as hydrophobic interactions, hydrogen bonds, van der Waals interactions, and electrostatic interactions, control the folding and association behavior of peptides and the conjugate as a whole. These non-covalent interactions are fairly weak and are on a similar energy level as those seen with polymer interactions and the entropy associated with polymer chain configuration. Upon conjugation of amorphous polymers to peptides, it is important to understand the effect on peptide structure and function and the nature of the polymer chain conformation. There are many questions that remain to be answered for such a complex, multicomponent system. Overall, such variables as the nature of the polymer, the complexity of the peptide, and the architecture of the conjugate must be understood for the realization of controlled assemblies. This may lead to specific design of the peptide sequence and the polymer block as needed.
Figure 1.5. Motivation behind research in the field of peptide-polymer conjugates can be divided into three categories: fundamental science, self-assembly, and applications (both biomedical and non-biological).

The second concerns the self-assembly of peptide-polymer conjugates in solution, in thin films, in the bulk, and at polar/nonpolar interfaces. A much sought after goal is to generate hierarchical nanostructures that display complex functionality. By combining peptides with synthetic polymers, multiple levels of hierarchical self-assembly are possible, in which the peptide block provides diverse chemical functionality. It may be important to control the localization or orientation of the functional peptide motif in various assemblies. For example, in order to take advantage of possible transport properties of peptides in thin films, it is important to orient them normal to the film. In addition, if they are to be used as signaling motifs on the surface of nanoparticles in solution, it is important that they be presented on the surface. In accordance with studies focused on fundamental science, a quantitative understanding of the protein sequence-structure-function relationship is required, along with their interactions with the polymer. These studies will provide valuable feedback and guidance in developing design principles for peptide-polymer conjugates. In addition, they can lead to novel materials with properties similar or superior to those found in nature. Addressing these areas may
bridge the gap between fundamental science and application and lead to the development of peptide-polymer conjugates suitable for both biological and non-biological applications.

Lastly, it is possible to generate functional nanoparticles based on these building blocks for a wide variety of applications, as depicted in Figure 1.6, because of the synergistic properties afforded by the two constituents. A vast majority of the work to date has focused on therapeutic applications, such as drug delivery, imaging, tissue engineering, and new vaccine formulation. As drug delivery is the prime motivation behind the later work in this dissertation, a more detailed discussion of the field of nanomedicine will be provided in Chapter 6. Furthermore, controlling the assembly of functional peptides that are capable of signaling, enzymatic reactions, or transport should allow for the realization of nanoreactors or compartments with controlled interior environments. Conjugating hydrophobic polymers will significantly enhance the processability of the peptide-polymer conjugates and expand the library of matrices to embed the peptides, in particular those derived from or designed based on transmembrane proteins. This may potentially lead to hybrid membranes with unique transport properties with high integrity in aqueous solution. The amphiphilic peptide-polymer conjugates may also form vesicles with helix bundles embedded at the interfaces and lead to man-made reactors mimicking those seen in cells. Furthermore, peptides assembled in thin films could also be used in filtration for the separation of molecules with high specificity. The possibilities are seemingly endless, as we have at our disposal the diverse, complex functions that nature has evolved and the many advancements made through synthetic chemistry.

**Figure 1.6.** Potential applications of peptide-polymer conjugates. Though possibilities exist in both biological and non-biological applications, a vast majority of the work to date has focused on therapeutic applications.
§ 1.5 General approach and synopsis of subsequent chapters

The work described in this dissertation has been a natural progression through the three motivating factors, starting with fundamental science in Chapters 2, 3 and 4, to self-assembly in Chapter 5, to finally a demonstration of a biomedical application, namely drug delivery, in Chapter 6. The search for fundamental understanding of the effects of conjugating polymers to peptides subsequently led to a rational design of optimal hybrid building blocks suitable for drug delivery applications. By understanding the various peptide intramolecular, peptide intermolecular, and peptide-polymer interactions that take effect, these hybrid materials can be tailored as desired to yield properties of interest. Chapters 2 through 4 are the focus of these fundamental studies upon variance of the nature of the polymer and the site of its conjugation. In Chapter 5, the understanding developed in the early chapters was applied toward the rational design of micelles suitable for drug delivery. Lastly, the fundamental characterization and preliminary biological evaluation of the micellar system are described in Chapters 5 and 6, respectively, demonstrating the unique opportunities afforded by these materials.
Chapter 2

Understanding the Effects of Conjugating Water-Soluble Polymers to Coiled-Coil-forming Peptides

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In this chapter, a new design of peptide-polymer conjugates is presented, where a water-soluble polymer is covalently linked to the side chain of a coiled-coil helix bundle-forming peptide. This novel architecture is compared to the typical linear diblock architecture, where a polymer chain is conjugated to a peptide terminus. The effect of conjugated polymers on peptide structure was examined using a de novo designed 3-helix bundle and a photoactive heme-binding 4-helix bundle. Upon attachment of poly(ethylene glycol) to the exterior of the coiled-coil, peptide secondary structure was stabilized and the tertiary structure was retained. Using a heme-binding peptide as an example, the new peptide-polymer conjugate architecture also preserves the built-in functionalities within the interior of the helix bundle. It is expected that the conjugated polymer chains act to mediate the interactions between the helix bundle and its external environment. Thus, this new peptide-polymer conjugate design strategy may open new avenues to macroscopically assemble the helix bundles and may enable them to function in non-biological environments. The preservation of peptide structure and function displayed here, accomplished solely by varying the site of conjugation, is key to further utilizing these hybrid materials in the generation of functional nanostructures.

§ 2.1 Introduction

As discussed in the preceding chapter, peptide-polymer conjugates have the potential to combine the advantages of synthetic polymers and peptides and can lead to hierarchically ordered, functional soft materials.1-12 Various types of peptide-polymer conjugates have been designed and investigated. Most are linear block copolymers and can be divided into two families, based on the complexity of the peptide sequences and the specificity of the intra- and inter-peptide interactions. The first family consists of an amino acid homopolymer or polypeptide as one block and a synthetic polymer as the second block. In both solution and the solid state, the polypeptide typically forms secondary structures and, upon phase separation of the block copolymer, assembles within microdomains, resulting in hierarchical assemblies with sub-nanometer features.6,10,11 Although specific interactions, such as electrostatic interactions and hydrogen bonds, have been introduced between each block to direct and manipulate the conjugate assemblies, the peptide’s built-in functionalities associated with a unique, designed sequence are lost with the use of amino acid homopolymers. The second family of peptide-polymer conjugates contains a specific peptide sequence with tailored intra- and inter-peptide interactions. Peptides forming α-helices and β-sheets, and higher order tertiary structures, have been used as building blocks.1,4,5,7,9,13-19 With these, hierarchical assemblies with molecular level control over chemical heterogeneity have been achieved. There have been, however, limited studies on peptide-polymer conjugates that utilize the built-in functionalities of coiled-coils to achieve high selectivity, specificity or responsiveness to external stimuli, as observed in natural proteins.
Despite the chemical and structural diversity provided by de novo designed peptides, they are limited by stability and degradation encountered during handling and are not amenable to standard fabrication processes that generate technologically important functional materials. Upon conjugating synthetic polymers to coiled-coils and obtaining proper assemblies, the synthetic polymers could act to protect, deliver and template the peptides on the nanometer scale, while the peptide bundles could provide molecular level control over the chemical heterogeneity to trigger, direct and execute built-in functionalities.\textsuperscript{1,3,32} The success in achieving desirable, functional assemblies relies on fundamentally understanding the interactions between each building block and delicately balancing and manipulating these interactions to achieve targeted assemblies without interfering with coiled-coil formation or designed functionalities.

Peptide-polymer conjugates, using PEGylated coiled-coil-forming peptides, were recently reported.\textsuperscript{7,14,20,45} The water-soluble conjugates were constructed by covalently linking PEG to the peptide N-terminus, forming essentially linear block copolymers. The presence of PEG was shown to enhance the stability of the peptide secondary structure against temperature and pH, acting as a shield between the peptide and its external environment. In the case of highly helical, long peptides, 35 and 42 amino acids in length, respectively, attaching a PEG chain to the N terminus did not affect the helical content nor interfere with homodimer formation.\textsuperscript{20} However, for a shorter \textit{de novo} designed coiled coil peptide, 23 amino acids in length, this architecture markedly decreased the helical content at peptide concentrations higher than 20 µM and lowered the degree of association.\textsuperscript{14,45}

It is possible that the attachment of PEG to the N-termini of the helical bundle restricts the volume available to the polymer chains, leading to molecular crowding and, thus, favoring lower aggregation states, as well as the unwinding of the ends of the helices, as depicted in Figure 2.1a. This effect becomes more obvious as the peptide’s oligomeric state and the polymer molecular weight increase and the peptide length decreases. Examination of the molecular models of helical bundles suggest that PEG attachment to the sides of the bundle, rather than to an end, as shown schematically in Figure 2.1b, would lead to less steric occlusion. In this way, the peptide secondary structure and also tertiary structure would be retained. Additionally, attaching the polymer chains to the side of the helical bundle would allow both the peptide termini and the interior channel of the bundle to be more accessible.

We have designed and studied a new family of coiled-coil-forming peptide-polymer conjugates, with the polymer chain covalently linked to the peptide side chain. Upon attaching poly(ethylene glycol) (PEG) chains to the exterior of a previously \textit{de novo} designed 3-helix bundle, the peptide secondary structure is stabilized. Also, the presence of PEG does not interfere with the peptide tertiary structure, i.e. the coiled-coil helix bundle. More importantly, using a photoactive, heme-binding, 4-helix bundle-forming peptide as an example, this new design preserves the built-in functionalities within the interior of the helical bundle. This design strategy for peptide-polymer conjugates opens a new avenue toward generating functional biomaterials that use more
sophisticated peptide structures to achieve high selectivity, sensitivity and responsiveness, as seen in nature. With the unique architecture of this designed peptide-polymer conjugate, the peptide structure is retained and both termini of the peptide are accessible, making it feasible to generate hydrogels with tailored spatial distributions and aggregation states of chemical motifs for tissue engineering. More importantly, the polymer chains on the exterior of the helical bundle could provide a handle to mediate interactions with the external environment, could potentially enable the macroscopic self-assembly of the helix bundles, and could allow the helix bundle-based machineries to function in non-biological environments. With the chemical and structural diversity of either naturally existing or de novo designed helical bundles and the many advantages of synthetic polymers, the prospect of fabricating materials with novel properties, superior to natural materials, is clearly possible.

§ 2.2 Results and Discussion

Here, we present a new design of helical bundle-forming peptide-polymer conjugates by covalently linking a polymer chain to the peptide side chain. Figure 2.1b shows the helical wheel of a typical de novo designed 3-helix bundle. The peptide primary structure is characterized by a heptad periodicity, -abcdefg-. Helical bundle formation is driven by the hydrophobic interactions between amino acids at positions a and d of each helix, forming a hydrophobic core. The bundle is further stabilized by the salt bridges between amino acids at positions e and g of adjacent helices. We chose the polymer conjugation site to be the amino acid at position f in the middle of the peptide sequence, as shown schematically in Figure 2.1c, to minimize any potential interference with helical bundle stability.

Two families of peptide-PEG conjugates have been synthesized based on previously de novo designed coiled-coil helix bundle-forming peptides. The first peptide, 1CW, is based on a known peptide sequence, 28 amino acids in length, called “1coi” in the protein data bank. The design of 1coi is very similar to the peptide sequences previously used to construct diblock copolymer type peptide-PEG conjugates. 1coi forms a coiled-coil 3-helix bundle that is stabilized by nonpolar valines and leucines at positions a and d, respectively, and Glu-Lys salt bridges at positions e and g, respectively. Gly-Trp-Asp-Gly-Arg (GWDGR) was added to the N-terminus of 1coi for future study. Serine at position 14 (f position on the helical wheel) was mutated to cysteine to facilitate conjugation of maleimide end-functionalized PEG. The helical wheel of 1CW, shown in Figure 2.1, depicts the arrangement of amino acids along the length of the helix. The second peptide studied, known as H10H24, is based on the diheme cytochrome b subunit of cytochrome bc1, as depicted in Figure 2.2. To conjugate end-functionalized PEG, the lysine at position 14 (f position on the helical wheel) was mutated to cysteine. The helical wheel of H10H24 is provided in Figure 2.3, which also depicts the arrangements of amino acids along the length of the helix. PEG
chains of three molecular weights, 750, 2,000 and 5,000 Da, were used to construct the peptide-PEG conjugates.

**Figure 2.1.** Schematic drawings of two designs of helical bundle peptide-polymer conjugates using a coiled-coil 3-helix bundle-forming peptide as an example: (a) polymers are conjugated to the end of the helical bundle, potentially creating steric hindrance and disrupting helical bundle formation; this effect becomes more obvious as the peptide’s oligomeric state and the polymer molecular weight increase and the peptide length decreases; (b) top view, depicting the helical wheel, and side-view of the new design of peptide-polymer conjugates, with the polymer attached to the side chain of the amino acid in the middle of the peptide sequence; (c) schematic drawing of the new peptide-polymer conjugate design, with polymer chains conjugated to the exterior of the helical bundle, potentially stabilizing helical bundle formation.

Peptide-PEG conjugates were purified by RP-HPLC and their identities and purities were verified by MALDI-TOF mass spectrometry. Figure 2.4 shows the mass spectrum of 1CW-PEG2K. The distance between two neighboring peaks is 44.05 Da, which is the mass of an ethylene glycol repeat unit. The lone peptides are perfectly monodisperse, so only the chain length heterogeneity of PEG contributes to the molecular weight distribution of the conjugate. In the MALDI-TOF mass spectrum, a representative
peak is labeled with its mass, which corresponds to the sum of the masses of an integer number (42) of ethylene glycol units and the mass of the peptide segment. This confirms the chemical integrity of the conjugates. The mass spectra of the other peptide-polymer conjugates of varying PEG molecular weights can be found in the Appendix (A.1).

Figure 2.2. H10H24 is an antiparallel coiled-coil 4-helix bundle derived from the cytochrome b subunit of cytochrome bc1. It is capable of binding 4 hemes per bundle via bis-histidyl ligation.

Figure 2.3. Helical wheel of H10H24 depicting the arrangement of amino acids along the length of the helix. The 14th amino acid at the f position of each peptide was mutated to cysteine to facilitate PEG conjugation.
Figure 2.4. MALDI-TOF mass spectrum of 1CW-PEG2K. The distance between two neighboring peaks is 44.05 Da, the mass of an ethylene glycol repeat unit. The labeled peak corresponds to the sum of the masses of an integer number (42) of ethylene glycol units and the mass of the monodisperse peptide segment.

Characterizing the effects of the conjugated polymer chains on the structure and functionality of the helical bundle is key in defining the utility of the peptide-polymer conjugate assemblies. The secondary structures of the peptide and the peptide-PEG conjugates were investigated by circular dichroism (CD). Figure 2.5a shows the CD spectra of ~30 μM solutions of 1CW, 1CW-PEG750, 1CW-PEG2K and 1CW-PEG5K in potassium phosphate buffer at pH 8. All traces show typical alpha-helix characteristics, with minima at 222 nm and 208 nm and a maximum around 195 nm. This data is evidence that PEG conjugation, for all molecular weights studied, enhances the helical content of the peptide. In addition, peptide helicity increases with an increase in PEG molecular weight: from ~63–66% for 1CW, to ~72% for 1CW-PEG750, to ~80–83% for 1CW-PEG2K and 1CW-PEG5K. In the case of linear diblock copolymer type peptide-PEG conjugates, a noticeable loss in helical content is observed, as shown in Figure 2.5b. The CD spectra comparing 1CW-PEG2K end conjugates to the unmodified 1CW and 1CW-PEG2K side conjugates clearly show a decrease in helicity upon conjugation of PEG to the N-terminus of the peptide. For other families of coiled-coils, the presence of PEG promoted helix formation for relatively short sequences (23 amino acids) only at low concentrations (< 20 μM), and subsequently reduced the helical content at higher concentrations.\textsuperscript{14} PEG chains of larger molecular weight displayed a greater decrease in peptide helicity. For relatively longer peptides (33 and 42 amino acids), the conjugated PEG did not affect the helical content.\textsuperscript{14,20,45} In this study, conjugating PEG to the exterior of the helical bundle (33 amino acids) does indeed stabilize the secondary
structure for all peptide concentrations studied, ~2 μM to 200 μM, and higher molecular weight PEGs, up to 5000 Da, do not unwind the helices.

**Figure 2.5.** (a) CD spectra of ~30 μM solutions of 1CW, 1CW-PEG750, 1CW-PEG2K, 1CW-PEG5K in 25 mM KH₂PO₄ buffer at pH 8 and 25°C. All traces exhibit typical alpha-helix characteristics. (b) CD spectra comparing ~30 μM solutions of 1CW and 1CW-PEG2K end and side conjugates. The PEG end conjugate slightly decreases the helicity of the unmodified peptide, whereas the side conjugate substantially enhances the helical content of the peptide.

Furthermore, the secondary structure of the conjugates at different pH values was investigated. The concentration dependence of the percent helicity of 1CW and 1CW-PEG2K in pH 3, 5, 8, and 11 buffers is plotted in Figure 4. For all pH values, peptide helicity increases with increased peptide concentration. At pH 3 and 5, the peptide exhibits high helical content, ~81%, at peptide concentrations above 20 μM, for both cases with and without conjugated PEG. At higher pH values (pH 8 and 11), the helicities of both the peptide and the conjugate decrease. However, the conjugates consistently display higher helical contents in comparison to their peptide counterparts. Conjugating PEG has the similar effect on helicity as reducing the pH. To exemplify, 1CW-PEG2K at pH 8 has a helical content near that of 1CW at pH 5. The common beliefs of the origin of enhanced helicity are that 1) PEG provides a microhydrophobic environment and/or 2) the presence of PEG introduces osmotic pressure that stabilizes the peptide/protein structure. Previous studies by H. Strey et al have clearly shown that, using PEG as an osmolyte, osmotic pressure can be used to induce a random coil-to-helix transition in poly(glutamic acid) and has a similar effect to that of reducing the pH. In this case, the experimental results show similar effects, although PEG is now covalently linked to the peptide. Since we do not know the spatial arrangement of the conjugated PEG chains relative to the peptide, the exact origin of the enhanced percent helicity is not
immediately clear. In particular, the superposition between the effect of conjugated PEG and that of the pH is unknown.

**Figure 2.6.** Concentration dependence of percent helicity of 1CW and 1CW-PEG2K in pH 3, 5, 8, 11 buffers at 25°C. The solid markers represent 1CW-PEG2K at the following pH: (●) pH 3, (▼) pH 5, (■) pH 8, and (▲) pH 11. The corresponding unfilled markers represent 1CW at each respective pH.

**Figure 2.7.** CD temperature melts of 30 μM solutions of 1CW, 1CW-PEG750, 1CW-PEG2K, and 1CW-PEG5K in pH 8 buffer.
The change in thermal stability of the peptide secondary structure upon PEG conjugation was investigated by taking temperature dependent CD measurements. Figure 2.7 shows the percent helicity as a function of temperature for 1CW, 1CW-PEG750, 1CW-PEG2K, and 1CW-PEG5K. Similar to what was seen at room temperature, the conjugated PEG stabilizes the peptide helices. The peptide-PEG conjugates display significantly higher helical contents than the peptide itself at all temperatures below 80°C. At temperatures below 60°C, 1CW-PEG5K displays a higher percent helicity than 1CW-PEG2K. This may be due to the greater presence of structured water molecules associated with the higher molecular weight PEG.\textsuperscript{47, 48} There, however, does not appear to be a difference in the melting temperatures of the conjugates in relation to the peptide itself. All samples have a melting temperature in the range of 70 - 80°C.

The effect of the conjugated PEG chains on helical bundle formation was determined using analytical ultracentrifugation (AUC). Fig 2.8 shows the sedimentation equilibrium analysis for 1CW, 1CW-PEG2K and 1CW-PEG5K in pH 8 buffer. The self-organization of 1CW was best fit as an ideal species, yielding a fitted molecular weight of 10177 Da and an oligomeric state of 3. 1CW-PEG2K and 1CW-PEG5K were best fit as ideal species, yielding fitted molecular weights of 17147 Da and 25069 Da, respectively. These molecular weights also correspond to oligomeric states of 3. For all peptide concentrations investigated (30 μM, 60 μM and 120 μM), 1CW-PEG2K and 1CW-PEG5K exhibit no distribution of monomers, dimers, or larger aggregates. Thus, the presence of PEG clearly does not interfere with the intended 3-helix bundle formation.

**Figure 2.8.** AUC sedimentation equilibrium radial absorbance profiles of 30 μM, 60 μM, 120 μM solutions of (left) 1CW, (middle) 1CW-PEG2K, and (right) 1CW-PEG5K at speeds of 20K, 30K, 40K rpm. Raw data are shown as symbols and their global fits as solid lines. The residuals for each fit appear above the radial absorbance profiles. The fitting of 1CW and its conjugates agree with single ideal species of 3-helix bundles.
Although CD spectra and AUC data both indicate that PEG does not deteriorate the peptide secondary or tertiary structures in this new design, it is unclear whether PEG may locally deform the peptide near the conjugation site and destroy an existing binding pocket in the interior of the bundle, which is essential for its designed functionality. To address this concern, cofactor binding can be used to assess the integrity of the helix bundle, especially for the purpose of generating functional biomolecular materials using peptide-polymer conjugates.

**Figure 2.9.** AUC sedimentation equilibrium radial absorbance profiles of 30 μM, 60 μM, 120 μM solutions of (left) H10H24 and (right) H10H24-PEG2K at speeds of 20K, 30K, and 40K rpm. Raw data are shown as symbols and their global fits as solid lines. The residuals for each fit appear above the radial absorbance profiles. The nonintegral fitting of H10H24 and its conjugates agree with previous work, indicating the presence of 4-helix bundles.

The second peptide studied, known as H10H24, is based on the diheme cytochrome b subunit of cytochrome bc1. H10H24 contains two histidine binding sites at positions 10 and 24 and forms a coiled-coil 4-helix bundle that can bind up to four hemes per bundle via bis-histidyl ligation. Each PEG chain is conjugated to cysteine at position 15 (f position on the helical wheel). H10H24 itself already displays a high helical content of ~81%. Upon conjugating PEG2K, only minimal changes in the helical content were observed (from ~81% for H10H24 to ~82% for H10H24-PEG2K). This is similar to the results of the diblock copolymer-type peptide-PEG conjugates with longer peptide sequences. Figure 2.9 shows the sedimentation equilibrium analysis of H10H24 and H10H24-PEG2K. For H10H24, the best fit of AUC data yielded a molecular weight of 12642 Da, which corresponds to an apparent oligomeric state of 3.4,
instead of the intended 4-helix bundle. This nonintegral degree of association has been observed previously; it was attributed to deviations in the partial specific volume of the protein complex from ideality because the sedimentation curves were independent of concentration over a wide range, and crosslinking the chains with disulfide bonds failed to change the apparent degree of association. Similarly, in the case of H10H24-PEG2K, the best fit yielded a molecular weight of 19260 Da and an oligomeric state of 3.36. Nonetheless, from the following heme-binding studies, we were able to conclude that the conjugated PEG does not interfere with helical bundle formation nor disrupts the cofactor binding sites.

Figure 2.10. (a) UV-Vis spectra of heme titrations into a 4 µM solution of H10H24-PEG2K recorded in a 1 cm path length cuvette, upon addition of 0, 0.8, 1.7, 2.5, 3.3, 4.1, 5.0, 5.8, 6.6, 7.5, 8.3, 9.1, 10.0 equivalents of heme per four-helix bundle. A vertical line indicates the peak at 412 nm. The dissociation constants were determined from the absorbance at 412 nm vs. the [heme]/[four helix bundle] ratio, as shown in the inset.

The effect of PEG conjugation on the interior of the helical bundle was investigated by studying heme titrations of H10H24, H10H24-PEG2K and H10H24-PEG5K. Figure 2.10 shows the UV-Vis spectra of the titration of a 200 µM solution of heme in DMSO into an aqueous solution of 4 µM H10H24-PEG2K. Agitation results in facile incorporation of heme into the peptides, as evidenced by an increase in the Soret peak at 412 nm and poorly resolved Qα and Qβ bands at 560 and 529 nm, respectively. As more heme was added, the blue-shift in the peak, due to the absorbance of free heme in solution, was observed. Figure 2.10 inset plots the absorbance at 412 nm as a function of
the heme to helix bundle ratio for H10H24-PEG2K. The change in slope of data sets for conjugated and non-conjugated peptides occurs at the same stoichiometry of heme to four-helix bundle, indicating that there is no substantial deleterious consequence of PEG conjugation. The heme titration experiments for H10H24-PEG5K also showed similar results. Multi-cofactor binding is a complicated phenomenon to quantify, so explicit determination of $K_{d1}$, $K_{d2}$, $K_{d3}$, and $K_{d4}$ was not possible. Based on this data coupled with previous studies, H10H24-PEG2K, similar to H10H24, shows $K_{d1}$ to be less than 1 nM and $K_{d2}$ to be in the range of tens of nM, both of which are not measurable at this peptide concentration. We suspect no significant change in the first and second binding sites upon PEG conjugation and possibly slight interference with the third and fourth binding sites, based on the heme titration data fitting. Details on titration data fitting can be found in the Appendix. Nonetheless, $K_{d3}$ for H10H24 and H10H24-PEG2K were both in the μM range. Figure 2.11 compares the overlay of the UV-Vis spectra of H10H24, H10H24-PEG2K, and H10H24-PEG5K, upon additions of heme yielding heme : bundle ratios of 1:1, 2:1, 3:1, and 4:1. The UV-Vis spectra of the holo-forms of H10H24-PEG2K and H10H24-PEG5K are essentially identical to that of H10H24, indicating that conjugated PEG does not interfere with the hydrophobic environment of heme, thus allowing retention of the designed functionality.

Figure 2.11. UV-Vis spectra of heme titrations into 10 μM solutions of H10H24, H10H24-PEG2K and H10H24-PEG5K recorded in a 1 cm path length cuvette, upon addition of 0, 1, 2, 3, and 4 equivalents of heme per four-helix bundle.
§ 2.3 Conclusion

In summary, a new family of coiled-coil helix-forming peptide-polymer conjugates has been constructed by covalently linking PEG to the exterior of the helix bundle. Two types of de novo designed coiled-coil helix bundles were investigated, and it was shown that conjugated PEG chains promoted helix folding and retained coiled-coil associations. In this way, self-assembly of the peptides was greatly enhanced, and thermal stability was retained. In the case of a photoactive heme-binding peptide, PEG maintained the integrity of the binding sites and did not interfere with cofactor binding within the interior of the helix bundle.

This new peptide-polymer conjugate design strategy could be extended to other coiled-coil peptides. With this architecture, the peptide terminus is accessible and the peptide can be modified via various chemical motifs with tailored spatial arrangements and aggregation states. This allows the use of the important properties of coiled-coil helical bundle-based hydrogels for tissue engineering. The accessibility of both of the peptide termini could also enable the anchoring of a peptide-polymer conjugate monolayer onto a substrate. Additionally, polymer chains conjugated to the exterior of the helical bundle could provide a handle to mediate interactions between the bundle and its external environment, and allow the helical bundle-based machinery to function in non-biological environments. The polymer chain could also mediate the inter-helical bundle interactions, lead to their macroscopic assemblage, and consequently, achieve macroscopic responses of the peptides’ built-in functionalities. In conjunction with recent advances in de novo protein design, the newly designed architecture of helical bundle-forming peptide-polymer conjugates presented here clearly shows great promise as a new avenue for peptide-based biomolecular functional materials.

§ 2.4 Experimental section

2.4.1 Synthesis

Two peptides, referred hereafter as 1CW (Ac-EVEALEKKVAALESKVQALEKKVEALEHG WDGR-CONH₂) and H10H24 (Ac-GGGEIWKLHEEFLKKFEELKLMHEERLKKM-CONH₂) were investigated. The peptides were synthesized on an AAPPTEC Apex 396 solid phase synthesizer using standard 9-fluorenethylmethyl carbamate (Fmoc) protection chemistry on Wang resin (Nova Biochem), typically at 0.3 mmol scale. The side chain protecting groups were as follows: Lys(Boc), Glu(OtBu), Asp(OtBu), Cys(Trt), Arg(Pbf), His(Trt), Trp(Boc), Ser(tBu), Gln(Trt). For the synthesis of 1CW-PEG conjugates, the serine at position 14 was mutated to cysteine to facilitate conjugation of maleimide end-functionalized PEG. Similarly for H10H24, the lysine at position 15 was mutated to cysteine. Prior to peptide cleavage from the resin, the N-terminus was acetylated using a 1:1 (v/v) acetic anhydride:
pyridine solution for 30 min. The peptides were cleaved from the resin and simultaneously deprotected using 90:8:2 trifluoroacetic acid (TFA)/ethanediol/water for 3.5 hr. Crude peptides were precipitated in cold ether and subsequently dissolved in water and lyophilized. Maleimide end-functionalized PEG, purchased from Rapp Polymere (Germany), was then coupled to the cysteine residues of the peptides, which were in white powder form, in 25 mM potassium phosphate buffer at pH 8 for 1 hour.3 PEGs of three varying molecular weights were utilized: 750, 2000, and 5000 Da. These are referred henceforth as PEG750, PEG2K, and PEG5K, respectively.

2.4.2 Reversed-phase high-pressure liquid chromatography

Peptides and their conjugates were purified by RP-HPLC (Beckman Coulter) on a C18 column (Vydac). Samples were eluted with a linear AB gradient, where solvent A consisted of water plus 0.1% (v/v) TFA and solvent B consisted of acetonitrile plus 0.1% (v/v) TFA. For purification of 1CW and its conjugates, the linear AB gradient of 37 to 42%B over 25 min was used, with typical elution of 1CW between 38-39%B and that of 1CW-PEG750, 1CW-PEG2K, and 1CW-PEG5K between 40-41%B. For purification of H10H24, typical elution was ~42%B on a gradient from 41 to 45%B over 20 min. H10H24-PEG2K and H10H24-PEG5K eluted at ~39%B on a 30 to 40%B gradient over 30 min. Peptide elution was monitored with a diode array detector at wavelengths of 220 nm and 280 nm. The flow rate was 10 ml/min for semi-preparative runs and peptides were injected at a concentration of 10-20 mg/mL.

2.4.3 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

The identity and purity of the peptides were verified by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry using α-cyano-4-hydroxycinnamic acid matrix. Mass spectra were recorded on an Applied BioSystems Voyager-DE Pro.

2.4.4 UV-Vis

Peptides were dissolved in buffered aqueous solution containing 25 mM potassium phosphate (KH2PO4) at pH 8 and 100 mM potassium chloride (KCl). Spectra were recorded on a Hewlett-Packard 8453 spectrophotometer using a standard 1 cm path length quartz cuvette. Peptide concentrations in solution were determined by their absorption at 280 nm due to each peptide’s lone tryptophan residue, assuming an extinction coefficient of 5500 M–1 cm–1, and using the Beer-Lambert Law.

Titration experiments were done using UV-Vis spectroscopy with various concentrations of H10H24, H10H24-PEG2K and H10H24-PEG5K solutions in quartz cuvettes. Spectra were recorded after addition of each aliquot, either 0.5 or 1 µL, depending on the concentration of hemin solution in DMSO, typically 200–500 µM, and the concentration of the peptide solution. The dissociation constant, Kd, of each heme binding site was determined by monitoring the shift of the heme absorbance at 412 nm as a function of the ratio of heme to 4-helix bundles and assuming extinction coefficients of
120,000 M$^{-1}$cm$^{-1}$ for bound heme and 35,000 M$^{-1}$cm$^{-1}$ for free heme.$^{22}$ $K_d$ was calculated as $K_d = [A][B]/[AB]$, where $[A]$ is the concentration of free peptide in solution, $[B]$ is the concentration of free heme in solution, and $[AB]$ is the concentration of heme bound to H10H24. This specific titration experiment was repeated four times to ensure reproducibility. Detailed descriptions of the evaluation of the heme dissociation constants can be found in the Appendix (A.1).

2.4.5 Circular dichroism

Circular dichroism measurements for secondary structure characterization were made on a Jasco J810 spectropolarimeter. CD spectra of each sample were recorded 3 times and averaged by collecting data from 260 to 190 nm at 0.2 nm intervals, a rate of 20 nm/min, a response time of 4 s, and a bandwidth of 1 nm. Samples were dissolved in 25 mM KH$_2$PO$_4$, 100 mM KCl buffer of various pH (3, 5, 8, 11). 1 mm path length quartz cuvettes were used for peptide concentrations ≥ 10 µM, and 2 cm cuvettes for concentrations < 10 µM. Ellipticity was reported as the mean residue ellipticity ([θ], deg cm$^2$ dmol$^{-1}$) and calculated as $[\theta] = [\theta]_{obs}/(10dcn)$, where $[\theta]_{obs}$ is the measured ellipticity in millidegrees, d is the optical path length in cm, c is the concentration of the sample in mol/L, and n is the number of amino acids. The mean residue ellipticity for a 100% helical peptide of infinite length was taken to be -37,400 deg cm$^2$ dmol$^{-1}$. Temperature melt measurements were made of ~30 µM solutions of peptides dissolved in pH 8 potassium phosphate buffer in 1 mm path length quartz cuvettes. The ellipticity was monitored at 222 nm as the temperature increased from 5°C to 95°C in 5°C increments, with a 1 min equilibration time at each temperature before the measurement was taken.

2.4.6 Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed on a Beckman Optima XL-A at 25°C with samples solubilized in 25 mM KH$_2$PO$_4$, 100 mM KCl buffer at pH 8. The path length of the cells was 1.2 cm and the An-60Ti rotor was used. Measurements at 20,000, 30,000, and 40,000 rpm were taken after 9 and 10 h of spinning at each speed to ensure equilibrium, which was verified by matching the early and late data sets. The radial distribution of absorbance was monitored at 280 nm. Sample concentrations were ~30, 60, 120 µM, and sample volumes were 120 µL. To prevent the formation of disulfide bonds that result in the dimerization of 1CW in pH 8 buffer, 100 mM of tris(2-carboxyethyl)phosphine (TCEP) was added to the peptide solution. The specific volume of 1CW was calculated to be 0.7496 mL/g using the software Sednterp (http://www.jphilo.mailway.com) and those of 1CW-PEG2K and 1CW-PEG5K were calculated to be 0.7785 mL/g and 0.7972 mL/g, respectively, by a weighted average of specific volumes between the peptide and PEG (0.833 mL/g). Similarly, the specific volumes of H10H24 and H10H24-PEG2K were calculated to be 0.7627 mL/g and 0.7827 mL/g, respectively. The density of the buffer was 1.004 g/mL. Nonlinear global fits were made using the software program Heteroanalysis (http://www.biotech.uconn.edu).
Chapter 3
Solution Structural Characterization of Peptide-PEG Side-Conjugates
Detailed structural characterization of protein-polymer conjugates and understanding of the interactions between covalently attached polymers and the biomolecules will build a foundation to design and synthesize hybrid biomaterials. Conjugates based on simple protein structures are ideal model systems to achieve these ends. Here, we present a systematic structural characterization study of coiled-coil peptide-PEG conjugates in solution using circular dichroism, dynamic light scattering, and small angle X-ray scattering to determine experimentally the conformation of conjugated PEG chains. The overall size and shape of helix-bundle-polymer conjugates were determined using a cylindrical form factor model. Detailed information regarding the covalently attached PEG chains were extracted using a newly developed model where each peptide-PEG conjugate was modeled as a Gaussian chain attached to a cylinder, which was built up to form bundles. Based on fitting of SAXS profiles, the peptide-polymer conjugates were found to retain helix bundle structure, with the polymer slightly compressed in comparison to the conformation of free polymers in solution. Such detailed structural characterization of the peptide-polymer conjugates, which elucidates the conformation of conjugated PEG around the peptide and assesses the effect of PEG on peptide structure, will contribute to the rational design of this new family of soft materials.

§ 3.1 Introduction

Peptide/protein-polymer conjugates are a promising class of hybrid soft materials that have the potential to synergistically combine the advantages of their individual constituents, while minimizing their respective liabilities. Peptides provide the precise chemical, structural, and functional specificity that synthetic polymers lack, while synthetic polymers provide biomolecules with improved processibility, solubility, and stability against temperature, pH, organic solvents, and degradation. Studies in the field of peptide-polymer conjugates have spanned the range from fundamental science (understanding and controlling the interactions between the two components), to self-assembly of these building blocks (for the generation of hierarchical, biofunctional nanostructures), to biological (most notably PEGylation of therapeutic proteins) and non-biological applications.

The most commonly used polymer in hybrid biomaterials is poly(ethylene glycol) (PEG). PEGylation has proven to be an effective strategy to enhance the kinetic stabilization of proteins, to obtain catalytic activity at very high temperatures, and to improve the stability, pharmacokinetics, and biodistribution of therapeutic proteins. FDA approved PEGylated proteins include Adagen, Oncaspar, Krystexxa, PEGASYS, and PEG-INTRON, among others. The unique chemical properties of PEG, such as its high solubility, amphiphilicity, and inertness, are keys to its effectiveness. Most studies have focused on the improved pharmacological
performance of PEGylated proteins compared to their unmodified counterparts and their differences in bioactivity. Little is actually known about the conformation of PEG when attached to a biomolecule. Detailed structural characterization of conjugates and understanding of the interactions between PEG and the biomolecule in solution remain lacking.

PEG's interaction with proteins has been investigated in several instances to determine its conformation. Currently, there are mainly two different models concerning the conformation of PEG when conjugated to proteins, both of which have various supporting claims in the literature. The most commonly presumed structure of PEGylated proteins is a shroud model, where the attached PEG chain wraps around the protein to create a shielding effect.\textsuperscript{28,32,33} Studies that indicate reduced antigenicity of proteins upon PEGylation assume a shroud model, as this is not expected if the majority of the protein surface is unmasked. A shroud model was also presumed in the analysis of a size exclusion chromatography study, where it was hypothesized that PEG forms a continuous, dynamic, flexible layer surrounding the protein.\textsuperscript{32} Furthermore, a small angle X-ray scattering (SAXS) study of PEG-antibody conjugates revealed a shroud model based on \textit{ab initio} modeling, where the two 20 kDa polymers are thought to effectively cover part or all of the antibody.\textsuperscript{33} These observations support the view that PEG interacts with proteins to form an exterior shell. In an alternate model, however, PEG does not interact significantly with the protein. Rather, the conjugate forms a dumbbell-like architecture, where two non-interacting entities are covalently linked.\textsuperscript{34,35} In a recent small angle neutron scattering (SANS) study that investigated the configuration of a 20 kDa PEG chain conjugated to lysozyme and human growth hormone, respectively, a dumbbell configuration was observed.\textsuperscript{34} From contrast variation studies, a relatively unperturbed PEG random coil was found adjacent to the globular proteins, with a radius of gyration ($R_g$) ~30% larger than that measured of free PEG in solution.\textsuperscript{34} It was supposed that the configurational entropy of a nonadsorbing end-conjugated polymer would be elongated relative to the unconjugated chain. In another study, PEG of molecular weight 5 kDa was conjugated to human galectin-2, which forms a dimer with the resultant conjugate containing 2 PEG chains. At low concentrations, the $R_g$ of conjugated PEG was found to be similar to that when free in solution. Furthermore, a model that combines the dumbbell and shroud views was observed in a PEG-hemoglobin study, where multiple PEG chains of molecular weight 5 kDa were conjugated to the protein.\textsuperscript{35} \textit{Ab initio} modeling of SAXS data indicated that a portion of PEG chains is bound to the surface of the protein and enters cavities between protein subunits, while the rest of the PEG chains moves away from the surface to yield an elongated structure. These conflicting observations found in the literature may be due to the fact that the properties of a PEG-protein conjugate depend on a variety of factors, such as the site of conjugation, the molecular weight of PEG, the number of PEG chains per protein, and the chemical heterogeneity of the surface of the protein. Furthermore, extraction of such information from an experimental point of view is challenging, even with scattering techniques, and clear conclusions may be difficult to make.
Engineering minimalist protein structures that self-assemble and reproduce native-like function, thus simplifying complex, globular proteins to relatively short, precisely folded peptides, is a valuable strategy for incorporating biomolecular building blocks to generate useful materials.\textsuperscript{36} They are also ideal model systems for developing a basic understanding of peptide/protein-polymer interactions and obtaining structural information for each component. In Chapter 2, a new design of peptide-polymer conjugates was described, where a polymer is conjugated to a side-chain in the middle of a coiled-coil-forming peptide.\textsuperscript{12} Here, relatively short peptides can self-assemble into a coiled-coil to form a simplified version of a globular protein that is more robust and better-defined in structure, while still being able to perform the specific function of the native protein, such as enzymatic activity, signal transfer and redox chemistry.\textsuperscript{37,38} This unique architecture, termed side-conjugates, contrasts the typical linear diblock copolymer-type, termed end-conjugates, where a polymer is conjugated to a peptide terminus. For end-conjugates, the energy associated with polymer chain deformation as a result of molecular crowding at the end of the helix bundle acts to unfold the peptide helix and disrupt coiled-coil formation. Side-conjugates effectively minimize steric hindrance between polymer chains. For side-conjugates based on water-soluble polymers such as PEG, the secondary structure was enhanced and the tertiary structure and functionality within the interior of the helix bundle were retained.\textsuperscript{12} Molecular dynamic simulations of side-conjugates further confirmed helix stabilization upon PEG conjugation.\textsuperscript{39} The oxygens of PEG were shown to interact favorably with the cationic lysine side chains on the exterior of the helix bundle.

This chapter presents a systematic structural study, using circular dichroism, dynamic light scattering, and SAXS, of coiled-coil peptide-PEG side conjugates in solution to determine experimentally the conformation of PEG when covalently attached to helix bundles. Two families of side conjugates, based on 3- and 4-helix bundles, respectively, were investigated. SAXS profiles were first analyzed using the Guinier approximation, and a cylinder form factor was used to determine the overall size and shape of the side-conjugates as a function of the molecular weight of PEG. In order to garner more detailed information about the structure of the conjugates and the polymer chain configuration in particular, the SAXS profiles were fit to a theoretical model, where coiled-coil side-conjugates were modeled as discrete ensembles of three or four cylinders, each attached with a Gaussian chain, thereby describing a bundle structure with polymers grafted to the sides.\textsuperscript{43} Based on fitting this scattering model to the SAXS profiles, the peptide-PEG side conjugates were found to retain helix bundle structure, with the PEG chain slightly compressed in comparison to the conformation of free PEG in solution. Such detailed structural characterization of the peptide-PEG conjugates will help elucidate the conformation of PEG around the peptide and provide structural guidance to determine the effect of PEG on peptide structure. Studies here can be readily extended to other peptide-polymer conjugate systems to provide basic structural information required for the implementation of these materials in hierarchical biofunctional nanostructures or as therapeutics.
§ 3.2 Small angle scattering

A schematic of the experimental geometry of a small angle scattering experiment in shown in Figure 3.1. A monochromatic beam is incident on a sample and scattering is collected on a 2D detector in transmission. The scattering angle is roughly a couple degrees and below. The scattered wavevector $q$ is the difference between the scattered wave and the incident wave and is related to the wavelength of radiation and the scattering angle by the equation, $q = \frac{4\pi \sin(\theta/2)}{\lambda}$. The ideal form factors of a cylinder and a core-shell sphere are included in Figure 3.1 because both of these models will be used to fit various structures throughout this dissertation. The 1D curves are generated by taking the radial integration of the 2D scatter.

![Small angle scattering schematic](image)

**Figure 3.1.** Schematic of the experimental geometry of a small angle scattering experiment. Resultant form factors of model cylinders and core-shell spheres are shown as examples.

![Scattering length density](image)

**Figure 3.2.** Comparison of scattering length densities of various atoms in the periodic table when using X-rays or neutrons as probes.
Small angle scattering can be done using either neutrons or X-rays as probes. Though both techniques oftentimes provide the same information, especially for our purposes, the physical mechanism of scatter is different because neutrons interact with the nuclei, while X-rays interact with the electrons of a sample. This gives rise to the difference in scattering length density (SLD) behavior of the two techniques, as depicted in Figure 3.2. The scattering length density of X-rays increases sequentially with atomic number because it is proportional to the electron density of atoms. Therefore, X-rays are not very sensitive to light atoms, and there is very little contrast between elements adjacent to each other on the periodic table. Neutrons have the advantage when probing soft materials, which are mostly composed of carbons, nitrogens, and oxygens, because their scattering length densities vary erratically between neighboring elements in the periodic table and between isotopes of the same element. In particular, the difference in SLD between isotopes makes it possible to tune the contrast by exchanging hydrogens with deuteriums in a specific component of a multicomponent system. Though SANS data will not be presented in this chapter, as SAXS is the main focus here, SANS will be presented in Chapter 5, and contrast variation could be utilized in the continuation of the work described here.

§ 3.3 Small angle scattering data analysis

The SAXS data presented in this chapter will be analyzed in three ways. First, to garner the overall radius of gyration of each conjugate, Guinier approximation was used. SAXS profiles were then fit to simple cylindrical form factors to determine the overall size and shape of the conjugates. Lastly, to decouple the polymer chain configuration from the structure of the coiled-coil, a new theoretical model was developed by Dr. Reidar Lund. Each of these analyses will be described in brief here.

3.3.1 Guinier approximation

The Guinier approximation provides the radius of gyration, \( R_g \), of the scattering particle. For low values of the scattering vector, \( Q \), \( R_g \) can be extracted from the slope of \( \ln I \) versus \( Q^2 \), as

\[
\ln I(Q) = \ln I(0) - Q^2 \left( \frac{R_g^2}{3} \right)
\]

in the limit of \( QR_g \ll 1 \). The scattering length density (SLD) was calculated based on the density measurements in this work or previously published data.\(^{42}\) At 25°C, the SLD, \( \rho \), of 1CW and H10H24 was determined to be \( 1.27 \times 10^{11} \text{ cm}^{-2} \). The SLD of PEG was taken to be \( 1.11 \times 10^{11} \text{ cm}^{-2} \), based on a density of 1.2 g/cm\(^3\),\(^{42}\) while that of the buffer containing 25 mM KH\(_2\)PO\(_4\) was calculated to be \( 9.43 \times 10^{10} \text{ cm}^{-2} \).
3.3.2 Modeling to a cylindrical form factor

Peptide-PEG conjugates were fit to a monodisperse cylindrical form factor of uniform scattering length density to determine the overall size and shape of the conjugates. The function is described as: $P(q)_{cy} = 2 J_1(qR \sin \alpha) \sin(qL \cos \alpha)$, where $A(q, \alpha)_{cy} = \frac{2J_1(qR \sin \alpha)}{qR \sin \alpha}$, where $R$ and $L$ are the radius and length of the cylinder, $\alpha$ is its angle to the scattering vector $q$, and $J_1(x)$ is the first order Bessel function. The integral over alpha averages the form factor over all possible orientations of the cylinder with respect to $q$. Data fitting was conducted using the SANS data analysis software provided by NCNR NIST.\(^{43}\)

Known information about sample scattering length density (SLD), solvent SLD, and the expected shape and size of the native peptide were implemented in model fitting. By using such constraints in the fits, the number of fitting parameters and the number of possible solutions were reduced by the elimination of many unphysical solutions.

3.3.3. Analytical bundle form factor modeling

In order to analyze the scattering data in more quantitative detail, we employed a model describing side-conjugates as cylinders with polymer chains grafted to the sides, where the cylinders assemble into bundles with oligomeric states of either 3 or 4. This model, which will henceforth be referred as the analytical bundle form factor model, will be described in detail in a separate publication.\(^{44}\) Briefly, scattering from a cylinder, which represents an $\alpha$-helix, with a single attached polymer chain that follows Gaussian statistics, was first considered. Using results originally developed for spherical and cylindrical block copolymer micelles,\(^{45,46}\) scattering from a single peptide-polymer conjugate is described as:

$$I(Q)_{chain-cyl} = V_{chain}^2 \Delta \rho_{chain}^2 P(Q)_{chain} + V_{cyl}^2 \Delta \rho_{cyl}^2 P(Q)_{cyl} + 2V_{chain} V_{cyl} \Delta \rho_{chain} \Delta \rho_{cyl} S(Q)_{chain-cyl}$$

(Eq. 1)

where $A(Q)_i$, $P(Q)_i$, $V_i$ and $\Delta \rho_i$ are the scattering amplitude, the form factor, the volume, and the contrast in SLD of either the chain or the cylinder ($i = chain/cyl$). Assuming a Gaussian chain for the polymer, $P(Q)_{chain}$ can be approximated using the Debye equation: $P(Q)_{chain} = 2(e^{-y} - 1 + y)/y^2$, where $y = (QR_g)^2$ and $A(Q)_{chain} = (1-e^{-\delta})/\delta$. The form factor of a cylinder takes the well-known form as described above. The interference term is given by assuming that the center-of-mass of the polymer is preferably located at a certain distance $\delta R_g$ away from the edge of the cylinder of radius $R$. Here $\delta$ is a numerical scaling factor ($\delta > 0$). This gives the following expression for a single side-conjugate: \(^{44,46}\)

$$S(Q)_{chain-cyl} = A(Q)_{chain} \int_0^{\pi/2} J_0(Q \cdot (R + \delta \cdot R_g) \sin \alpha) \frac{\sin(QL \cos \alpha/2)}{QL \cos \alpha/2} \cdot A(q, \alpha)_{cy} \sin \alpha d\alpha$$
In order to construct the coiled-coil side-conjugates, the center-to-center distance between two parallel cylinders is $fR$, where $f$ is a swelling factor that regulates the distance between the cylinders. For solid cylinders that cannot interpenetrate, $f \geq 1$. A polymer chain is grafted to the side of each cylinder. Neglecting correlations between polymers conjugated to different peptides, the corresponding expression is the “structure factor” of the conjugate: $S(Q) = \frac{1}{N_{cyl}} \sum_{i,j} J_0(Qd_{ij})$, where $J_0$ is the zero-order Bessel function, $N_{cyl}$ is the number of cylinders in each bundle and $d_{ij}$ is the distance between centers of the cylinders. This description has also been applied to glucagon fibrils.

In principle, this expression can be used to calculate the correlations between any number of parallel cylinders of any configuration. Here, we restrict ourselves to three special cases: a 2-helix bundle, a trigonal (equilateral) 3-helix bundle, and a square 4-helix bundle. In these cases, the following expression are obtained:

$$S(Q)_\text{bundle} = \begin{cases} 
\frac{1}{4} (2 + 2J_0(2QRf)) & 2\text{-helix} \\
\frac{1}{9} (3 + 3J_0(2QRf)) & 3\text{-helix} \\
\frac{1}{16} (4 + 8J_0(2QRf) + 4J_0(\sqrt{8}QRf)) & 4\text{-helix}
\end{cases}$$

The scattering from an arbitrary mixture of 2-, 3-, and 4-helix bundles, together with dissolved single helix peptide-polymer conjugates of fractions $f_2$, $f_3$, $f_4$, and $(1-f_2-f_3-f_4)$, respectively, can then be written as:

$$I(Q)_\text{bundle} = \frac{\phi \cdot I(Q)_{\text{chain-cyl}}}{V_{\text{peptide}} + V_{\text{chain}}} \left[ (1-f_2-f_3-f_4) + 2f_2S(Q)_\text{bundle}^{(2)} + 3f_3S(Q)_\text{bundle}^{(3)} + 4f_4S(Q)_\text{bundle}^{(4)} \right]$$

(Eq.2)

where $\phi$ is the total volume fraction. This equation has the correct dimension of cm$^{-1}$. Note that this expression can also be used to describe non-PEGylated peptide bundles by setting the $V_{\text{chain}}=0$.

§ 3.4 Results and discussion

3.4.1 Varying the molecular weight of PEG: 1CW

Two families of peptide-PEG side conjugates were investigated, one based on a de novo designed 3-helix bundle, 1CW (1coi in the Protein Databank), and the other based on a naturally derived heme-binding 4-helix bundle, H10H24. The molecular weight of PEG was varied, from 1 kDa, 2 kDa, and 5 kDa. Schematics of both families of side-conjugates, based on 1CW and H10H24, respectively, are shown in Figure 3.3. As discussed in Chapter 2, PEG side-conjugation of coiled-coils led to full retention of
peptide structure and function and stabilization against variations in pH and temperature.\textsuperscript{12} Here, a systematic structural characterization study of coiled-coil peptide-PEG side-conjugates in solution is presented, where circular dichroism (CD), dynamic light scattering (DLS), and small angle X-ray scattering (SAXS) were used to determine experimentally the conformation of conjugated PEG chains and the form factor of the conjugates.

\textbf{Figure 3.3.} Schematic of peptide-PEG side-conjugates based on 1CW (top), a de novo designed three-helix bundle, and H10H24 (bottom), a heme-binding four-helix bundle.

In Chapter 2, CD experiments were carried out at low concentrations (~30 μM) to assess the effect of PEG conjugation on the secondary structure of the peptide.\textsuperscript{12} To confirm peptide structure and stability of the SAXS samples, CD was performed under similar conditions (high concentration, ~300 μM) as those of the SAXS measurements. For all samples, CD spectra showed well-defined α-helical structures with high helicity. The effect of varying the molecular weight of PEG on the secondary structure of 1CW side-conjugates was first assessed as a function of temperature. Data shown in Figure 3.4a indicates that PEG side-conjugation leads to an increase of ~20–35\% in α-helicity of the peptide at all temperatures, consistent with previous findings. This can be attributed to osmotic pressure introduced by the presence of PEG that stabilizes peptide folding, similar to what has been observed for polypeptides in the presence of free PEG in solution.\textsuperscript{53} The increase in concentration increases the melting point of each sample, from the range of 70–80°C at low concentrations to >85°C at high concentrations. This is
consistent with previous studies where the thermal stability of an oligomeric system increases with increasing concentration,54,55 and the fact that the melting temperature depends on protein concentration indicates the existence of intermolecular cooperativity in the peptide folding process.56,57 Based on the CD results, we fully expect the conjugates measured by SAXS to retain their peptide secondary structures. Furthermore, the ratio of the CD signals at 222 nm and 208 nm can be used to assess the tertiary structure of the peptide. A ratio $\theta_{222}/\theta_{208} \geq 1$ indicates the presence of coiled-coils, whereas $\theta_{222}/\theta_{208} \leq 0.86$ indicates isolated helices.58,59 The coiled-coil 1CW retains a $\theta_{222}/\theta_{208} \geq 1$ below 45°C and unfolds fully at ~85°C. Upon PEG side conjugation, the ratio $\theta_{222}/\theta_{208}$ remains above 1 below 65°C and above 0.86 across the temperature range studied (Figure 3.4b). Therefore, the conjugated PEG serves to inhibit substantial α-helix unfolding and helix bundle uncoiling.

Figure 3.4. Temperature dependent circular dichroism of ~300 μM solutions of 1CW and 1CW-PEG side-conjugates of various PEG molecular weights. (a) Helical content of the peptide (b) $\theta_{222}/\theta_{208}$ of the peptide, which alludes to the tertiary structure of the peptide. A ratio $\theta_{222}/\theta_{208} \geq 1$ indicates the presence of coiled-coils, whereas $\theta_{222}/\theta_{208} \leq 0.86$ indicates isolated helices, as demarcated by the dashed lines.58 59 PEG side-conjugation serves to inhibit substantial α-helix unfolding and helix bundle uncoiling.

DLS, shown in Figure 3.5, was used to determine the hydrodynamic size of the coiled-coil side-conjugates in solution. DLS indicates that all samples form primarily individual bundles, rather than larger scale aggregates or individual monomers. The measured hydrodynamic radii ($R_h$) are listed in Table 3.1. A consistent increase in the hydrodynamic radius was observed as the molecular weight of PEG increases, from 16 Å for 1CW, to 18 Å for 1CW-PEG1k, to 22 Å for 1CW-PEG2k, and 27 Å for 1CW-PEG5k.
As a rough guide, the differences in $R_h$ between conjugates coincide with the estimated increases in size of PEG with molecular weight when in solution.\textsuperscript{60}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure3_5.png}
\caption{DLS of 1CW-PEG side-conjugates as a function of PEG molecular weight. The hydrodynamic sizes indicate formation of individual bundles for all samples, rather than larger scale aggregates or individual monomers.}
\end{figure}

Figure 3.6 shows the concentration normalized SAXS profiles (in units of mL/mg/cm) of a series of concentrations for (a) 1CW and (b) 1CW-PEG2k. The data do not exhibit any significant concentration dependences, indicating that there are no strong interactions between individual bundles that would be manifested in structure factor peaks or depression of the low-$Q$ scattering associated with a positive virial coefficient. As seen, the samples display a rather weak scattering pattern, with some noise at low-$Q$. Nevertheless, despite the low intensity and the noise in the data, a clear plateau is seen at low $Q$ (Guinier region), followed by a rather pronounced decay at high $Q$. This indicates that no significant large aggregates are present and that the structures are rather well-defined, in agreement with the DLS data. The high-$Q$ decay is observed for both samples, which can be approximated as a $Q^{-x}$ dependence, with a value $x > 2.5 - 3$. Though the decay for the PEGylated sample appears smoother, it is still significantly greater than that expected for polymer-like samples, which show a decay of typically $x \sim 1.7 - 2$.\textsuperscript{61} This is indicative of bundle formation rather than denatured peptides in solution, which would show polymer-like scattering.

The concentration normalized SAXS data of all 1CW-PEG conjugates of different PEG molecular weights are shown in Figure 3.7. The decay in the scattering profile gradually shifts towards lower $Q$-values with increasing PEG molecular weight, consistent with an increase in the overall size of the conjugates. This is accompanied by a concomitant increase in the “broadness” or “width” in the decay, which can be attributed
to increased polymer-character of the system as the volume fraction of the polymer increases. The forward scattering does not seem to depend much on PEG molecular weight in this $Q$-range. As will be seen in the analytical form factor model fits performed on absolute scale, this is an effect of a lowering of the overall contrast, which partly counterbalances the increase in molecular weight for low molecular weight ranges.

Figure 3.6. Concentration normalized scattered intensity (in units of mL/mg/cm) of a series of concentrations for (a) 1CW and (b) 1CW-PEG2k obtained using SAXS. The data do not exhibit any significant concentration dependences, indicating that there are no strong interactions between individual bundles nor significantly large aggregates present.
First, Guinier analysis of the SAXS profiles were performed in order to determine the overall \( R_g \) of each conjugate. The Guinier fits of each conjugate are included in the SI and the values are tabulated in Table 3.1. There is a general increase in \( R_g \) as the molecular weight of PEG increases. These values correspond well to the hydrodynamic radii measured by DLS, again indicating that the conjugates form individual bundles of roughly \(~4–5\) nm in overall size.

**Table 3.1**

<table>
<thead>
<tr>
<th>1CW</th>
<th>PEG MW</th>
<th>( R_h ) (DLS)</th>
<th>( R_g ) (Guinier)</th>
<th>( R ) (cylinder)</th>
<th>( L ) (cylinder)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>16</td>
<td>18 ± 0.5</td>
<td>12 ± 1</td>
<td>55 ± 5</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>18</td>
<td>18 ± 2</td>
<td>15 ± 1</td>
<td>52 ± 5</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>22</td>
<td>20 ± 0.5</td>
<td>14 ± 1</td>
<td>58 ± 5</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>27</td>
<td>27 ± 2</td>
<td>20 ± 1</td>
<td>60 ± 5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>H10H24</th>
<th>PEG MW</th>
<th>( R_h ) (DLS)</th>
<th>( R_g ) (Guinier)</th>
<th>( R )</th>
<th>( L )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>16</td>
<td>22 ± 2</td>
<td>14 ± 1</td>
<td>59 ± 5</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>18</td>
<td>17 ± 0.5</td>
<td>16 ± 1</td>
<td>48 ± 5</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>21</td>
<td>25 ± 1</td>
<td>15 ± 1</td>
<td>55 ± 5</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>32</td>
<td>31 ± 3</td>
<td>20 ± 1</td>
<td>60 ± 5</td>
</tr>
</tbody>
</table>

A simple cylindrical form factor of uniform electron density was first chosen as a model for fitting of the overall size and shape of the conjugates, as shown in Figure 3.7. Based on the crystal structures of 1CW and H10H24 found in the Protein Databank (PDB), this model could be used as a first approximation of the native coiled-coils. Measurement of the crystal structure of the unmodified 1CW coiled-coil yields a radius of \(~11\) Å and a length of \(~41\) Å, when modeled as a simple cylinder. These dimensions yield an \( R_g \) of \(~14\) Å (\( R_g^2 = R^2/2 + L^2/12 \) for a cylinder), which is comparable to the \( R_g \) determined by Guinier analysis and the \( R_h \) measure by DLS. The cylindrical fit of the 1CW helix bundle (Figure 3.7), which yields a radius of 12 Å and a length of 55 Å, is comparable to its crystal structure and was used as a basis of comparison for the scattering profiles of the PEG conjugates. The length is slightly longer than that expected from the crystal structure, possibly as a result of hydration of the peptide.

Though a cylindrical model is clearly a simplification of the PEG conjugates, it may nevertheless provide a suitable representation of the overall size and shape, as other possible geometrical shapes, such as prolate or triaxial ellipsoids, were also applied, with less success. In the fits, two parameters, \( R \) and \( L \), were allowed to vary. However in order to obtain reasonable fits for larger PEG molecular weights, the intensity prefactor, \( I(0) \), also had to be varied, i.e. the fits could not be performed on an absolute scale. While the model seems to work reasonably well for the unPEGylated system, the fit quality
progressively breaks down with increasing molecular weight of PEG, in particular at low-Q. As a result, the cylindrical model yields unphysical cylinder lengths, especially for the PEG5K conjugate, as seen in Figure 3.7, where the fit model is not able to describe the scattering contributions from the peptide bundle and the polymer simultaneously. Generally, upon fitting of the scattering profiles of the PEGylated samples to cylindrical form factors within a restricted Q-range, the length increases to ~60 Å, and the radius increases to ~15-20 Å. The increase in length may be attributed to the increase in helical content of the peptide upon PEG conjugation, as the random coil portion of the peptide allows solvent molecules to permeate, whereas the rigid helix does not. The increase in radius is expected with the addition of the PEG chains to the exterior of the bundle. Though the cylinder model provides a rough estimation of the overall size of the conjugates and confirms the formation of helix bundles, the fit quality is poor for larger molecular weights of PEG. We were unable to draw conclusions regarding the polymer chain conformation around the helix bundle. In order to garner insight into the configuration of PEG, a more detailed and realistic analytical bundle form factor model was developed to decouple the contribution of the polymer from the coiled-coil.

**Figure 3.7.** Concentration normalized SAXS profiles of 1CW-PEG samples of varying PEG molecular weights. Solid lines display a comparison to a simple cylindrical form factor. Poor fits were observed as the PEG molecular weight increases, indicating simple cylinder form factor fits are not sufficient to describe PEG chain conformation beyond certain molecular weights.
The analytical bundle form factor fit model (Eq. 1 & 2) was developed in order to gain more detailed information concerning the structure and aggregation behavior of the peptide-polymer conjugates and of the polymer chain configuration in particular. In order to obtain reliable and accurate results, the fits were made on an absolute intensity scale and all molecular details were included. In the model, it was assumed that the center-of-mass of the polymer, represented by Gaussian chains, is localized somewhere a distance $\delta R_g$ from the edge of the cylinder. No assumptions concerning any preferential angle or position along $L$ were made. This is clearly an oversimplification, but can be justified to a certain degree, as the polymer segments are likely to have a diffuse conformation delocalized along the bundle length. In order to take into account preferential conformation angles, more theoretical work needs to be made, which is outside the scope of this article. In any case, as seen from the fit quality shown in Figure 3.8, a very good description of the data is achieved with this simplified model.

![Graph](image)

**Figure 3.8.** Concentration normalized SAXS profiles of 1CW-PEG samples of varying PEG molecular weights. Solid lines display analytical bundle form factor model fits as described in the text. This model describes the conjugates well, yielding fits of the radius of individual helices (cylinders) to be $\sim 4.5 - 6$ Å and lengths of about $L \sim 40-50$ Å for all PEG molecular weights. The conformation of PEG conjugated to the exterior of coiled-coils was found to be compressed compared to that expected of PEG when free in solution.
In the first set of fits, the displacement of the peptide-polymer distance, \( \delta \), and the swelling factor between helices, \( f \), were kept equal to 1, as their values could not be determined unambiguously given the limited resolution of SAXS. The separation between each helix is thus given by \( d = 2R \). In the final fits, only \( R \), \( L \) and \( R_g \) were varied. The fits were performed on an absolute scale, including the experimentally determined densities (1.4 g/mL were estimated for both peptides) and molecular weights. However, in order to correct for small uncertainties with regard to concentration, etc., associated with working with small volumes, a prefactor on the order of 1 was applied to the overall intensity. In the fits, it was assumed that only 3-helix bundles were present at room temperature, i.e. \( f_3 = 1 \) (\( f_2 = f_1 = f_4 = 0 \)), consistent with AUC data already published. No clear evidence of smaller bundle structures, which would primarily be reflected in reduced forward scattering and a decay at higher \( Q \), was found at this temperature.

As seen in Table 3.2, which lists the dimensions garnered from the analytical form factor modeling fits, this model describes the data very well. The radius of individual helices (cylinders) was found to be on the order of 4.5 – 6 Å, which translates to an inter-helical distance of about 9 – 12 Å (the swelling factor, \( f \), was set to 1 in all cases). The length of the 1CW bundles was found to be about \( L \sim 40-50 \) Å for all PEG molecular weights. There is ~5-10 Å uncertainty in the length of conjugates from model fitting associated with low scattering signal, uncertainties related to parasitic scattering around the beamstop, and background subtraction. Fits of the pure 1CW peptides (\( R \sim 5.8 \) Å and \( L \sim 49 \) Å) were compared with the dimensions of its crystal structure (\( R \sim 5.5 \) Å, \( L \sim 41 \) Å). Whereas the radius coincides rather well, the length deduced from solution scattering seems to be slightly longer. Again, the apparently larger \( L \) could be attributed to partially unfolded ends of the peptide and/or hydration of the \( \alpha \)-helix leading to effectively larger lengths. In addition, there may be conformational changes within the bundle different from the crystal structure caused by hydration by water molecules. From this model, the conformation, i.e. the radius of gyration, of the polymer chains grafted to the coiled-coil can also be extracted. The following values are obtained for the 1CW-PEG conjugates: \( R_g \approx 8, 12 \) and 23 Å for PEG1k, 2k and 5k, respectively. A detailed discussion of the PEG conformation will be continued below.

When fitting to the analytical form factor model, the minimum number of free variables necessary to provide reasonable fits was utilized. As a consequence, only two variables were used for the bundle structure (\( R, L \)), while only one (\( R_g \)) was used for the polymer. The other parameters, such as \( f \), the factor which modulates the inter-cylindrical distance, \( d = 2Rf \), was found to be obsolete within the available resolution of the SAXS data and was set to \( f = 1 \). The distance from the cylinder wall to the center of the polymer chain was fixed to \( R_g \) in the final fits (\( \delta = 1 \)). However, it is possible that the preferential distance is either closer to the peptide (collapsed or shroud-like) or depleted away from the surface (dumbbell-like). This situation can, to a certain degree, be mimicked in the model by setting \( \delta < 1 \) and \( \delta > 1 \), respectively. However, this was used with caution, as small \( \delta \) yields unphysical interpenetration between the polymer and the peptide and large \( \delta \) results in unphysical degrees of chain stretching, as the polymer must remain bound to
the surface of the peptide. For these reasons, along with the ambiguity in the fits, where several $\delta$ and $R_g$ values could give equally good fit qualities, $\delta$ was fixed to 1.

Table 3.2

<table>
<thead>
<tr>
<th></th>
<th>PEG MW</th>
<th>R</th>
<th>d</th>
<th>L</th>
<th>$R_g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1CW</td>
<td>0</td>
<td>5.8 ± 1.0</td>
<td>11.6</td>
<td>49 ± 5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>6.1 ± 1.0</td>
<td>12.2</td>
<td>46 ± 5</td>
<td>8 ± 2</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>5.8 ± 1.0</td>
<td>11.6</td>
<td>51 ± 5</td>
<td>11 ± 2</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>5.5 ± 1.0</td>
<td>11.0</td>
<td>41 ± 5</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>H10H24</td>
<td>0</td>
<td>5.3 ± 1.0</td>
<td>10.6</td>
<td>50 ± 5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>6.1 ± 1.0</td>
<td>12.2</td>
<td>45 ± 5</td>
<td>8 ± 2</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>4.9 ± 1.0</td>
<td>9.8</td>
<td>55 ± 5</td>
<td>13 ± 4</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>4.6 ± 1.0</td>
<td>9.2</td>
<td>45 ± 5</td>
<td>25 ± 4</td>
</tr>
</tbody>
</table>

In order to illustrate the stability of the fits and the possible range of the parameters, calculations were performed while varying $\delta$, as shown in Figure 3.9a, using the data for 1CW-PEG5k as an example because it has the largest polymer contribution. As seen, the fit quality is rather insensitive to small variations in $\delta$ around $\delta = 1$, and many values are equally probable. Large $\delta$ represent unphysical dumbbell-like structures which are also not compatible with the experimental data. Therefore, values close to 1 are the most reasonable, which justifies a choice of $\delta = 1$ in the final fits. In the next set of calculations, shown in Figure 3.9b, $\delta$ was fixed to 1 and the radius of gyration, $R_g$, was varied. In this way, both the conformation of the polymer and the distance separating the peptide from the polymer are varied simultaneously. In order to facilitate the comparison, $R_g$ was varied with respect to the expected $R_g$ of free 5k PEG in water, which is about $R_g \sim 32$ Å. We thus define $\gamma = R_g/R_g(solv)$ and vary $\gamma$, as shown in Figure 3.9b. As expected, a rather strong dependence is found upon varying $\gamma$. Both small $\gamma$ (0.2 - 0.5) and large $\gamma$ ($> 1$) result in strong deviations. The best fit was obtained with $\gamma \approx 0.7$, indicating that the preferred PEG conformation is slightly smaller than that of the homopolymer when free in solution. Discussion of the polymer chain conformation will be revisited later.
Figure 3.9. Calculations of the analytical form factor model performed in comparison to the scattering profile of 1CW-PEG5k while varying (a) $\delta$ and (b) $\gamma$ to demonstrate the stability of the fits and the effects of the parameters. $\gamma = R_g/R_g(solv)$ where $R_g(solv)$ is the radius obtained from free PEG in solution estimated from previous experimental results.\textsuperscript{62}
3.4.2 Varying the molecular weight of PEG: H10H24

Similar studies were done for H10H24, a heme-binding four helix bundle that was also studied previously. PEG side conjugation was shown not to interfere with peptide secondary and tertiary structures and heme-binding functionality within the interior of the bundle. Dynamic light scattering was used to determine the hydrodynamic size of the conjugates in solution, as shown in Figure 3.10. Though H10H24 is a 4-helix bundle while 1CW is a 3-helix bundle, cylindrical models of their crystal structures yield similar dimensions. DLS also shows that both peptides have the same measured $R_h$. As expected, there is a steady increase in size as the molecular weight of the conjugated PEG increases, from 16 Å for the peptide alone, to 18 Å for H10H24-PEG1k, to 21 Å for H10H24-PEG2k, to 32 Å for H10H24-PEG5k. All H10H24 conjugates appear to form individual helix bundles rather than larger aggregates.

![Figure 3.10. DLS of H10H24-PEG side-conjugates as a function of PEG molecular weight. The hydrodynamic sizes indicate formation of individual bundles for all samples, rather than larger scale aggregates or individual monomers.](image)

SAXS of H10H24 and H10H24-PEG conjugates are shown in Figure 3.11. Similar to the case of the 1CW-PEG conjugates, the scattering patterns show clear evidence of particle-like scattering rather than polymer scattering. Guinier approximations of the $R_g$ of the conjugates are listed in Table 3.1. The $R_g$ of H10H24 was estimated to be 19 Å, similar to that of 1CW. Upon PEGylation, the $R_g$ generally increases with increasing molecular weight of the polymer and correspond well to the $R_h$ measured by DLS. The scattering profiles of H10H24-PEG conjugates were first fit to cylindrical form factors, as shown in Figure 3.11. Again, for samples that yielded
unphysical dimensions, fitting was restricted to higher $Q$-ranges. All conjugates display a cylindrical radius of \( \sim 15\text{-}20 \text{ Å} \) and a length of \( \sim 50\text{-}60 \text{ Å} \). The dimensions of the conjugates determined here indicate that coiled-coil formation is retained.

In complete analogy to the analysis described above for the 1CW-PEG conjugates, the scattering of H10H24-PEG conjugates was fit to the analytical form factor model using Eq. 2 and setting $f_4 = 1$ and all other fractions equal to zero. It was thereby assumed that 4-helix bundles, as previously determined from AUC, are predominant in solution.\(^{12}\) As seen in Figure 3.12, the model describes the data very well. Fitting of the pure H10H24 4-helix bundle results in $R \sim 4.5 \pm 0.5 \text{ Å}$ and an overall length $L \sim 50 \pm 5 \text{ Å}$. From the crystal structure available in the PDB, H10H24 has a radius $R \sim 5 \text{ Å}$ and a length $L \sim 45 \text{ Å}$. As was observed for 1CW, a larger length is obtained in solution, while the radius is similar. For PEGylated H10H24 samples, fits of $R \sim 3.8 - 5.6 \text{ Å}$ and $L \sim 45\text{-}55 \pm 5 \text{ Å}$ were obtained, along with the following values for the polymer: $R_g \cong 8, 13 \text{ and } 25 \text{ Å}$ for PEG1k, 2k and 5k, respectively.

![Figure 3.11](image.png)

**Figure 3.11.** Concentration normalized SAXS profiles of H10H24-PEG conjugates of varying PEG molecular weights. Solid lines display a comparison to a simple cylindrical form factor. Poor fits were observed as the PEG molecular weight increases, indicating simple cylinder form factor fits are not sufficient to describe PEG chain conformation beyond certain molecular weights.
Figure 3.12. Concentration normalized SAXS profiles of H10H24-PEG conjugates of varying PEG molecular weights. Solid lines display fits using the analytical form factor model as described in the text. This model describes the conjugates well, yielding fits of the radius of individual helices (cylinders) to be \( \sim 3.8 \) – 5.6 Å and lengths of about \( L \sim 45 \) - 55 Å for all PEG molecular weights. The conformation of PEG conjugated to the exterior of coiled-coils was found to be compressed compared to that expected of PEG when free in solution.

3.4.3 Conformation of PEG conjugated to peptide

The conformation of the PEG chain conjugated to the peptide is rather challenging to extract, in particular for low PEG molecular weights, because scattering is dominated by the peptide bundle. Computer simulations of peptide-PEG side conjugates indicate specific interactions between lysine side chains and PEG that result in the enhanced stability of peptide structure observed previously.\(^{39}\) This may perturb the conformation of PEG and lead to an asymmetric shape of the polymer coil, where certain segments are partially collapsed onto the peptide helix. Such effects are hard to accommodate theoretically and would, in the present fit model, only be manifested by a change in the overall conformation \( R_g \) and/or the distance between the cylinder wall and the PEG chain, \( \delta R_g \). As discussed earlier, reasonable fits were obtained using \( \delta = 1 \), i.e. the distance between the cylinder wall and the center of mass of the polymer was set...
equal to $R_g$. This led to $R_g$ values on the order of $10 – 30 \text{ Å}$, depending on the molecular weight of PEG, as listed in Table 3.3. In order to compare these data with that expected of free PEG in solution, the $R_g$ was estimated assuming Gaussian chains and from empirical scaling laws reported in the literature.\textsuperscript{62,63} For a Gaussian chain under theta-conditions, we may use $R_g^2 = N_{PEG} C_N n l_0^2 / 6$, where $N_{PEG}$ is the number of monomers, $n$ the number of bonds per monomer, $l_0 = 1.46 \text{ Å}$ is the mean bond length of PEG, and $C_N$ is the characteristic ratio that describes the flexibility of the chain. The latter is expected to depend on molecular weight and only assumes a constant asymptotic value at high molecular weights ($C_N \sim 5.5$). Table 3.3 compares the results using these values of $C_N / C_\infty$ to the estimations using the experimentally determined power laws. The latter approach, obtained from measuring $R_g$ of PEG of different molecular weights in the range of 1-1000 kg/mol\textsuperscript{63} (in one study) and 100-1000 kg/mol\textsuperscript{62} (in another) in water includes excluded volume interactions and takes the form $R_g \approx c M^\nu$, where $\nu \sim 0.6$ and $c$ is a numerical prefactor. It should be mentioned that for low molecular weight PEG, excluded volume effects are unlikely to be important and $\nu \sim 0.5$ is expected.\textsuperscript{66} With this in mind for the current range of molecular weights used in this study, the scattering model, which assumes Gaussian chains, can be expected to be a good approximation.

As seen in the comparison in Table 3.3, the respective radii of gyration of PEG determined for each conjugate all have values, within error, that are close to the values determined for PEG under theta-conditions. Furthermore, comparing the data for both 1CW and H10H24, the same values of $R_g$ are found, within experimental errors, indicating that the conformation of PEG is not very sensitive to the nature of the peptide when conjugated to the exterior of coiled-coils. When comparing them to the values estimated from empirical results of free PEG in water, the chain dimensions seem to be smaller in all cases. This is clearer for the higher molecular weights of PEG, where the values of free PEG assume values of $16 – 18 \text{ Å}$ for PEG2k and $28 – 32 \text{ Å}$ for PEG5k, while the values of PEG when conjugated to peptides yield values between $12 – 13 \text{ Å}$ and $24 – 25 \text{ Å}$ for PEG2k and PEG5k, respectively.

When closely evaluating the sequences of 1CW and H10H24, the cationic lysines on the surface of the coiled-coils, which were found to interact favorably with PEG from molecular simulations,\textsuperscript{39} are $\sim 10 \text{ Å}$ away from the middle of the peptide, or the site of PEG conjugation. This coincides with the minimal $R_g$ determined by analytical form factor model fitting. Therefore, it is possible that PEG interacts favorably with specific residues of the peptide, thereby decreasing its effective $R_g$ as compared to free PEG in solution, but does not create a shroud over the entire coiled-coil. Interactions between PEG and the polymer are also expected due to the increased thermal stability of the conjugate in comparison to the unmodified coiled-coil. It is suspected that the coiled-coil-PEG side-conjugates form a structure between that of the shroud-like model and the dumbbell model, similar to the PEG-hemoglobin study described earlier.\textsuperscript{35} A portion of
the PEG chain is bound to the surface of the coiled-coil, possibly through specific interactions with select residues of the peptide, while the rest of the PEG chain moves away from the surface and occupies a Gaussian conformation. It is possible that the different claims in the literature supporting either the shroud model or the dumbbell model are likely due to the differences in chemical heterogeneities of the protein surfaces, among other factors. It is expected that the chemical nature of the polymer and the chemical heterogeneity of the biomolecule affect how the two interact, and thus they determine the effective polymer chain conformation and the effect on peptide structure upon polymer conjugation, as observed in other studies.\textsuperscript{67,68}

| Table 3.3 |
|---|---|---|---|---|---|---|
| PEG MW | 1CW | H10H24 |
| R\textsubscript{g} ave. | R\textsubscript{g} ave. | R\textsubscript{g} 0(min)\textsuperscript{1} | R\textsubscript{g} 0(max)\textsuperscript{2} | R\textsubscript{g} (solv1)\textsuperscript{3} | R\textsubscript{g} (solv2)\textsuperscript{4} |
| 1000 | 8 ± 2 | 8 ± 2 | 10.3 | 11.6 | 12.5 | 11.1 |
| 2000 | 12 ± 2 | 13 ± 4 | 14.6 | 16.4 | 18.8 | 16.6 |
| 5000 | 23 ± 4 | 25 ± 4 | 23.1 | 25.9 | 32.2 | 28.2 |

1) Calculation based on $R\textsubscript{g}^2 = N\textsubscript{PEG} C_N n_0^2 / 6$, where $C_N = 4.5$

2) Calculation based on $R\textsubscript{g}^2 = N\textsubscript{PEG} C_N n_0^2 / 6$, where $C_N = 5.5$

3) Extrapolation using data from Devanand et. al.\textsuperscript{59}

4) Extrapolation using data from Kawaguchi et. al.\textsuperscript{60}

An assumption of the analytical form factor model is that the polymer conformation follows Gaussian statistics. Possible distortion and anisotropy of the PEG conformation due to specific interactions between PEG and certain amino acids along the helix bundle are not taken into account here. Such distributions might lead to a similar overall $R_g$, although the overall shape is anisotropic. These effects cannot be determined without more sophisticated measurements, e.g. contrast variation SANS, which are planned for future work.

\textbf{§ 3.5 Conclusions}

We have presented a systematic small angle X-ray study of a novel family of peptide-polymer conjugates based on the side conjugation of coiled-coils. The form factors determined here indicate the formation of coiled-coils upon polymer conjugation and a compressed conformation of PEG around the coiled-coil compared to that expected
of PEG when free in solution. Not only do these peptide-polymer conjugates present interesting fundamental questions about how the two components interact with one another, but they also provide tremendous promise for generating hierarchical, functional, self-assembled nanostructures with potential utility in both biological and non-biological applications. The information garnered here is important for future developments with these building blocks.

§ 3.6 Experimental section

3.6.1 Circular dichroism

Temperature dependent circular dichroism measurements were made using a Jasco J810 spectropolarimeter. The temperature was increased from 5° to 85°C in 5° increments, with a 1 min equilibration time at each temperature before the measurement was taken. CD spectra of each sample at each temperature were recorded by collecting data from 260 to 190 nm at 0.2 nm intervals. A rate of 20 nm/min, a response time of 4 s, and a bandwidth of 1 nm were used. Samples were dissolved in 25 mM KH₂PO₄ buffer at pH 7.4 and measured in 1 mm path length quartz cuvettes. Measurement of samples at the same concentration as those of the scattering experiments was not possible due to significant scatter at short wavelengths that reduced CD signal. Therefore, samples were measured at the highest concentration possible (~300 µM) while still achieving sufficient signal. Ellipticity was reported as the mean residue ellipticity ([θ], deg cm² dmol⁻¹) and calculated as [θ] = \frac{θ_{obs}}{10 dcn}, where [θ_{obs}] is the measured ellipticity in millidegrees, d is the optical path length in cm, c is the concentration of the sample in mol/L, and n is the number of amino acids. The mean residue ellipticity for a 100% helical peptide of infinite length was taken to be -37,400 deg cm² dmol⁻¹.

3.6.2 Dynamic light scattering

Dynamic light scattering was performed on the same samples prepared for the SAXS experiments using a Malvern Zetasizer Nano ZS. Concentrations ranged from ~0.5-1 mM. Samples were filtered through a 0.2 µm pore filter immediately prior to measurement.

3.6.3 Density measurements

The specific density of 1CW in solution was estimated using an Anton Paar DM 5000 Densiometer. The procedure involved measuring the density of a 1 vol% 1CW solution and the corresponding buffer. The specific solution density was found to be approximately 1.4 g/mL at 25°C.
3.6.4 Small angle X-ray scattering data collection

SAXS experiments were carried out at the SAXS/WAXS/GISAXS beamline 7.3.3. of the Advanced Light Source (ALS) at the Lawrence Berkeley National Laboratory (LBNL). The instrument was operated using an X-ray energy of 10 keV and a sample-detector length of 1.9 m, providing a $Q$-range of 0.001 to 0.4 Å\(^{-1}\), where

$$Q = \frac{4\pi}{\lambda} \sin\left(\frac{\theta}{2}\right), \quad \theta = \text{scattering angle}, \quad \lambda = 1.24 \ \text{Å}.$$ 

The instrument was equipped with a 1 M Pilatus detector.

The samples were contained in standard Boron-Quartz capillaries (Charles Supper) held in a customized "home-made" sample holder which permitted the sample to be filled and removed using a syringe. In this way, background subtraction could be made quantitatively and absolute intensity calibration was possible using water as a primary standard. All samples were dissolved in pH 7.4, 25 mM KH\(_2\)PO\(_4\) buffer at concentrations of ~0.5 – 1 mM, unless otherwise stated. Samples were filtered through 0.2 μm nylon filters and were monitored closely to account for possible beam damage. It was found that no radiation damage was visible for 5 s acquisition times. The absolute scattering intensity was also verified using a calibrated glassy carbon sample kindly provided by Dr. Jan Ilavsky at APS, ANL, Illinois.

Similar SAXS profiles were also collected using a home-made circulating flow cell, following a design previously described.\(^{41}\) In brief, the sample cell, made from 6061-T6 aluminum, has windows made from 25 μm thick potassium aluminosilicate (muscovite mica) sheets (Goodfellow) which scatter only very weakly. The mica is glued to the cell with 302-3M epoxy (Epoxy Technology) and reinforced with a quick dry epoxy. Solution samples were circulated through the cell using a peristaltic pump. Measurements were conducted in flow cells, in conjunction with the static capillary measurements, to confirm the lack of radiation damage to samples. As no significant differences were found between the scattering profiles measured in the two types of holders, only the SAXS profiles measured in capillaries will be presented here.
Chapter 4

Understanding the Effect of Conjugating Hydrophobic Polymers to Coiled-Coil-forming Peptides

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Engineering amphiphilicity into peptide-polymer conjugates may enable them to self-assemble into functional, biomolecular materials with great implications in both biological and non-biological applications. Like other amphiphilic molecules, such as lipids and synthetic block copolymers, amphiphilic peptide-polymer conjugates can self-assemble into various structures, such as spherical micelles, cylindrical micelles, and vesicles, when dissolved in a selective solvent for one block. The formed structure is dependent on the packing parameter of the amphiphile, so much is dependent on the sizes of the head and tail groups. Since the peptide structure determines the size, shape and intermolecular interactions of these building blocks, systematic understanding of how the peptide structure and functionality are affected upon implementing hydrophobicity is required to direct their assemblies in solution and in the solid state. However, depending on the peptide sequence and native structure, previous studies have shown that the hydrophobic moieties affect peptide structures differently. Here, we present a solution study of amphiphilic peptide-polymer conjugates where a hydrophobic polymer, polystyrene, is covalently linked to the N-terminus of a coiled-coil helix bundle-forming peptide. The effect of conjugated hydrophobic polymers on the peptide secondary and tertiary structures was examined using two types of model, coiled-coil helix bundles. In particular, the integrity of the binding pocket within the helix bundle upon hydrophobic polymer conjugation was evaluated. Upon attachment of polystyrene to the peptide N-terminus, the coiled-coil helices partially unfolded and functionality within the bundle core was inhibited. These observations are attributed to favorable interactions between hydrophobic residues with the PS block at the peptide-polymer interface that lead to rearrangement of peptide residues and consequently, unfolding of peptide structures. Thus, the hydrophobicity of the covalently-linked polymers modifies the conjugates’ architecture, size and shape, and may be used to tailor the assembly and disassembly process. Furthermore, the hydrophobicity of the covalently-linked polymer needs to be taken into consideration to maintain the built-in functionalities of protein motifs when constructing amphiphilic peptide-polymer conjugates.

§ 4.1 Introduction

Engineering amphiphilicity into peptide-polymer conjugates may enable them to self-assemble into functional, biomolecular materials with great implications in both biological and non-biological applications. A majority of studies have focused on hybrid block copolymers composed of typically hydrophobic synthetic polymers and water-soluble polypeptide segments that fold into α-helices. These methods allow various peptide functionalities to be incorporated into the cores of the self-assembled structures, which could potentially be useful in biomedical applications. In solution, these families of block copolymers based on polypeptides form a multitude of structures with tunable morphologies. Stimuli-responsive nanocapsules can be designed with molecular level interactions to achieve desired behaviors or functionalities.
control over their shape and size.\textsuperscript{10,15-17} In thin films and in the bulk solid state, a majority of conjugates tend to form hexagonal-in-zigzag lamellar morphologies, with the peptide helices either interdigitated or folded and packed hexagonally.\textsuperscript{18-24} On a much smaller length scale, diblock oligomers of similar compositions were found to form temperature-dependent morphologies, different from the lamellar structures seen with the higher molecular weight analogues.\textsuperscript{1,10,11} As a whole, this collection of work, based on amphiphilic polypeptide-polymer conjugates, demonstrates a rich library of hierarchical structures in solution and in the solid-state. Fine control over conjugate morphologies, including stimuli-responsiveness, can be achieved by tailoring the intra- and intermolecular interactions and the other energetic contributions involved in the assembly process.

A majority of studies have focused on hybrid block copolymers composed of typically hydrophobic synthetic polymers and water-soluble polypeptide segments that fold into $\alpha$-helices.\textsuperscript{15,89,90,93,96} In solution, these block copolymers based on polypeptides form a multitude of structures with tunable morphologies.\textsuperscript{97-99} For example, stimuli-responsive nanocapsules can be designed with molecular level control over their shape and size.\textsuperscript{4,90,100,101} In thin films and in the bulk, most conjugates tend to form hexagonal-in-zigzag lamellar morphologies, with the peptide helices either interdigitated or folded and packed hexagonally.\textsuperscript{90,93,96,102-108} This collection of work, based on amphiphilic polypeptide-polymer conjugates, demonstrates a rich library of hierarchical structures that can be formed in solution and the solid-state.

Much work has also been done with giant amphiphiles, compounds composed of an entire enzyme or protein as the headgroup and a hydrophobic polymeric tail attached to a preselected, well-defined location.\textsuperscript{25,26} In aqueous media, these giant amphiphiles, based on either the iron storage protein ferritin or the enzyme horse radish peroxidase (HRP),\textsuperscript{27-30} were able to form aggregate structures similar to those formed by conventional amphiphilic block copolymers, namely vesicles, micelles, and fibers of micellar rods.\textsuperscript{31-41} Triblock hybrid copolymer analogues have also been synthesized and were found to form rare structures, such as Y-junctions, toroids, octopus structures, dumbbells, and other aggregates, aside from the expected structures.\textsuperscript{42,43}

Most work on amphiphilic conjugates to date is based either on simple $\alpha$-helix forming polypeptides or complex full proteins/enzymes. Biomolecular building blocks that fall between the two aforementioned groups, such as protein tertiary structural motifs, may also provide much promise for the construction of functional, hybrid materials.\textsuperscript{44-46} Using a common tertiary motif, the coiled-coil, as an example, this minimalist protein structure simplifies complex, globular proteins. They can be readily used as stimuli-responsive motifs that are difficult to obtain using solely synthetic polymers. Leucine zippers have been used extensively as physical crosslinking points in generating hydrogels for tissue engineering.\textsuperscript{47-49} Recently, a heterodimer coiled-coil was used to noncovalently link poly(ethylene glycol) (PEG) and polystyrene (PS) blocks and the resultant amphiphilic PEG-peptide-PS triblock copolymer assembled into thermo-responsive micellar assemblies in aqueous solution that transformed from rodlike
micelles to spherical micelles upon heating. In addition to serving as crosslinkers, coiled-coils are desirable building blocks because they can retain certain functionalities of native proteins, such as transport, signaling and enzymatic reactions. Recent developments in de novo protein design also allow one to extend their functionalities further. From a materials point-of-view, these simplistic protein motifs are much more robust against temperature and solvents in comparison to their natural counterparts.

The structural simplicity and potential built-in functionalities of coiled-coil helix bundles, in conjunction with directed hierarchical assemblies and enhanced processibility upon implementing amphiphilicity via conjugation of hydrophobic synthetic polymers, could potentially lead to hybrid materials that exhibit novel properties. However, these hydrophobic moieties also affect the peptide structure that determines the size and shape of each building block, as well as their intermolecular interactions, and consequently, the phase behavior of the amphiphilic conjugates. By attaching alkyl tails to collagen-like peptides, intermolecular interactions between peptides lead to unique assemblies of peptide amphiphiles in solution. The helical structures of the collagen-like peptides were stabilized upon attaching hydrophobic alkyl tails, and their thermal stability was enhanced substantially. A similar effect was observed upon conjugating a PS chain to the N-terminus of a random coil peptide and was attributed to the formation of micelles in aqueous solution. However, for the same peptide that was blended with its complementary peptide helix to form a heterodimer, the presence of the hydrophobic polymer led to significant unfolding of the heterodimer. Decreased enzyme activity of Candida antarctica (CALB) was also observed upon covalently linking PS and was attributed to hydrophobic PS-induced destabilization of the active conformation of the enzyme. Coupling PS to heme, a cofactor of HRP and myoglobin (Mb), also disturbed heme binding in the apo-protein or reduced access of the substrate to the active site of the protein. In addition, extensive studies have shown that proteins adsorbed onto hydrophobic surfaces denature and lose their enzymatic activities due to hydrophobic interactions. These studies indicate that hydrophobic polymers affect protein folding differently for peptides/proteins that structure at multiple levels. For coiled-coils, such effects depend strongly on the periodicity of hydrophobic residues and on inter-helix interactions. The functionalities of coiled-coil helix bundles and the phase behavior of amphiphiles rely heavily on the protein structure at multiple levels and, in particular, on the integrity of the binding pocket. Thus, there is a great need to systematically investigate the effect that covalently linked hydrophobic polymers have on the peptide structure. This is of particular importance as we construct biomolecular materials using different families of protein motifs.

Here, using coiled-coil helix bundle-forming peptides as a model system, we present a solution study of amphiphilic peptide-polymer conjugates where a PS chain is covalently linked to the N-termini of a coiled-coil helix bundle. The present contribution mainly focuses on the peptide secondary and tertiary structures, as well as on the cofactor binding pocket in the interior of the helix bundle, in order to assess any changes to the
structure and functionality of the water-soluble coiled-coil upon hydrophobic polymer attachment. This fundamental understanding is important as we aim to build a foundation to direct assemblies of amphiphilic peptide-polymer conjugates based on tertiary protein motifs. It was found that the presence of a hydrophobic polymer partially unfolded the peptide secondary structure and consequently, compromised the binding pocket within the core of the coiled-coil due to hydrophobic polymer-peptide interactions at peptide/polymer interfaces. These studies show that imparting overall amphiphilicity to the conjugate by adding hydrophobic polymers may lead to hierarchical assemblies and raises the possibility for incorporation into polymer thin films or at polar/nonpolar interfaces. Present studies provide basics on how various parameters affect the building blocks and their assembly process, as well as the structure-property relationship of amphiphilic conjugates based on helix bundle-forming peptides, which could thereby lead to the development of design principles for generating biomolecular functional materials.

§ 4.2 Results and discussion

Two families of peptide-polymer conjugates have been prepared by end-conjugating PS (M<sub>n</sub> = 1,000 g/mol) or PEG (M<sub>n</sub> = 2,000 g/mol) to a model, de novo designed, coiled-coil 3-helix bundle and a naturally derived, heme-binding 4-helix bundle. Peptide-PEG conjugates were prepared to decouple the effects of steric hindrance and peptide-polymer interactions on the peptide structure. The two peptides, referred as 1CW and H10H24, are described in detail in a previous study. Using reverse phase-HPLC (RP-HPLC), resolution of conjugates differing in only the degree of polymerization of PS was achieved. This is different from that observed with water-soluble peptide-polymer conjugates. Figure 4.1a shows that the RP-HPLC chromatogram of crude H10H24-PS1K contains a series of peaks. Each peak, as identified by MALDI-TOF, corresponds to conjugates with a monodisperse PS chain. Figure 4.1b shows the MALDI-TOF spectrum for an isolated peak. The single mass is evidence that separation of the polydisperse conjugate by PS degree of polymerization was achieved, with the PS degree of polymerization increasing sequentially with elution time. Similar separation was also observed for 1CW-PS1K conjugates. All relevant peaks (n<sub>PS</sub> = 6-14) were combined for further characterization due to limited material.

Peptide-PS conjugates cannot be readily solubilized in buffered aqueous solution and form large aggregates. The optical turbidity prevents accurate determination of the peptide concentration and structure. A common nonionic surfactant, n-Octyl-β-D-Glucopyranoside (OG) was used to reduce the aggregate size and stabilize micellar assemblies. OG was selected because of its relatively high critical micelle concentration (CMC), 0.73 wt%, in comparison to many other nonionic surfactants, thereby enabling the solubilization of amphiphilic peptide-PS conjugates without forming surfactant micelles.
Figure 4.1. (a) RP-HPLC chromatogram of H10H24-PS1K (wavelength monitored at 280 nm). (b) MALDI-TOF mass spectrum of an isolated RP-HPLC peak. The single mass is evidence that separation of the polydisperse conjugate by polystyrene degree of polymerization was achieved, with the PS degree of polymerization (n) increasing sequentially with elution time.

Dynamic light scattering (DLS) and circular dichroism (CD) studies were first carried out to determine the proper OG surfactant concentration at which the presence of OG surfactant has minimal effect on the peptide structure. Three OG concentrations, 0.45, 0.9, 1.2 wt%, were investigated. These values were purposely selected to be below, around and above the CMC (0.73 wt%) of OG. DLS of 0.45 wt% OG buffer solution verified no micelle formation. The 0.9 and 1.2 wt% OG buffers alone showed OG micelles with a hydrodynamic diameter of ~4 nm. The effect of the presence of OG on the secondary structure of the unmodified peptide was first evaluated. Figure 4.2a shows the mean helicity of 1CW dissolved in buffer solution with different OG concentrations as a function of peptide concentration. Without OG surfactant, the helicity of 1CW plateaus to ~65%. DLS also shows that 1CW forms ~2.9 nm nanoparticles, as expected based on its crystal structure. At 0.45 wt% OG, the average helicity of 1CW also plateaus to ~65%. However, further increase of OG concentration to 0.9 and 1.2 wt% leads to a decrease in helical content.
Amphiphilic peptide-PS conjugates form aggregates of sizes in the range of hundreds of nanometers to a micron in buffer solutions. Adding OG surfactant solubilizes the conjugates and the solutions were optically clear for all OG concentrations considered. DLS was performed as a function of OG concentration to first determine the effect of the OG surfactant on the aggregation state of the conjugates. Figure 4.2b shows the DLS of 1CW-PS1K solutions with 0.45, 0.9 and 1.2 wt% OG. At 0.45 wt% OG, below its CMC, 1CW-PS1K conjugates form micellar structures, ~11.2 nm in size, where PS segregates itself from water to form the hydrophobic core and 1CW forms the outer shell. For the range of peptide-PS conjugates studied (5 µM to 200 µM), the molar ratio of OG to conjugate varied from 4000:1 to 100:1, with the OG surfactants serving to stabilize the formation of peptide-PS micelles. At 0.9 and 1.2 wt% OG, the size of the aggregates decreased to ~3.8 nm. Based on the peptide crystal structure, this should correspond to the size of an individual peptide-PS conjugate surrounded by OGs. Above the CMC of OG, the high concentration of surfactants present in solution is thought to encapsulate individual helices, thereby preventing aggregation between PS chains and consequently, coiled-coil helix bundle formation. Based on these CD and DLS studies, systematic studies on the effect of covalently linked hydrophobic polymers on the coiled-coil structure and binding pocket were conducted in solutions containing 0.45 wt% OG. Though it is noted that the conjugates formed micellar assemblies rather than being solubilized into individual coiled-coils at this surfactant concentration, the solution was optically clear and allowed for further structural and functional characterization of these amphiphilic coiled-coil-polymer conjugates.
Figure 4.3. CD spectra of ~50 μM solutions of 1CW, 1CW-PEG2K, and 1CW-PS1K in 25 mM KH$_2$PO$_4$ buffer at pH 8, with 0.45 wt% OG. All traces show typical α-helix characteristics.

Figure 4.3 shows the CD spectra of ~50 μM solutions of 1CW, 1CW-PS1K, and 1CW-PEG2K in 0.45 wt% OG, potassium phosphate buffer at pH 8. All traces show typical α-helix characteristics, with minima at 222 nm and 208 nm and a maximum around 195 nm. 1CW, the unmodified peptide, displays a helical content of ~65%. The helicity drops to ~55% for 1CW-PEG2K and ~40% for 1CW-PS1K. Polymer conjugation to the N-terminus decreases the helical content of the peptide, with a more drastic reduction observed with the hydrophobic polystyrene than with the water-soluble PEG. Attaching PEG to the N-terminus of similar peptides was shown previously to induce unwinding of peptide helices due to steric effects. Here covalently linking hydrophobic PS has much stronger deleterious effects and unwinds the peptide helices further, even though the molecular weight of PS is lower than that of PEG (1K Da vs. 2K Da). These results suggest that the hydrophobicity of the polymer may have a major impact on the peptide structure.

There have been studies on peptide secondary structures where hydrophobic moieties were linked to the N-terminus of coiled-coils. Peptide-amphiphiles with collagen-like peptides and dialkyl chain tails have shown to self-assemble into highly ordered coiled-coil structures, with the alkylation substantially stabilizing the triple-helix. In this case, the triple-helix consists of polypeptides with repeat sequences of (Gly-Pro-Hyp) that form left-handed polyPro II like helices. Recent studies by Marsden et al. focused on a heterodimer based on a heptad repeat peptide sequence. When conjugating PS to one helix of a heterodimer, the peptide was partially structured with increased helicity. This was attributed to the micellar structures in solution that increased the local peptide concentration. However, upon blending with the complimentary helix, the overall helicity of the heterodimer reduced significantly, from 74% to 33%, when the
PS block was covalently linked to one helix, even though the amphiphilic peptide-PS conjugates still formed micellar assemblies in solution.50

These results indicate that covalently linking a hydrophobic polymer to a coiled-coil helix bundle may be different from that of a single peptide helix. For coiled-coil helix bundles, hydrophobic moieties have different effects on the peptide structure and can lead to unfolding of peptide helices. The percentage of helix unwinding induced by the PS block is less for 1CW than that for the heterodimer reported previously and may be due to the longer peptide sequence of 1CW (33 vs. 22 amino acids) and stronger inter-helix interactions. We speculate that the peptide structure in an amphiphile depends on the peptide-polymer interactions at the peptide/polymer interface and on inter-helix interactions. It is likely that at the peptide/PS interface, peptide residues are arranged so that the hydrophobic residues preferentially interact with PS, with the peptide serving as a surfactant to minimize the interactions between the hydrophobic PS block and water, as shown schematically in Figure 4.4. For an unstructured peptide, this leads to peptide folding into a helix, as seen in studies by Marsden et. al. as well as that shown for peptides with a similar hydrophobic periodicity at an apolar/water interface.78 In the case of coiled-coil helix bundles, such as 1CW, the α-helix is amphipathic with a repeat heptad sequence of \textit{abcdefg}, where the \textit{a} and \textit{d} positions are typically hydrophobic amino acids. The favorable interactions between \textit{a} and \textit{d} residues with the PS block lead to unfolding of 1CW helices, as schematically shown in Figure 4.4. Peptide unfolding compromises helix bundle formation and reflects the energetic competition among peptide/PS interactions, inter-helix interactions and the energetic gain from burying hydrophobic residues in the interior of the coiled-coil. For amphiphiles based on collagen-like peptides, the peptides’ strong tendency to form 3-helix bundles exceeds the energetic gain from satisfying peptide/alkyl tail interactions. It is also likely that such an energetic gain is not as high as that seen with 1CW-PS since the alkyl tail is less hydrophobic than PS. The effect of the hydrophobicity of the conjugated polymers will be a subject of future studies.

![Figure 4.4](image.png)

**Figure 4.4.** Idealized schematic drawing of 1CW-PS1K, a linear diblock peptide-polymer conjugate based on a coiled-coil 3-helix bundle motif, and the effect of PS conjugation to
the N-terminus of the helix bundle-forming peptide. The conjugation of a hydrophobic polymer to the N-terminus of the peptide leads to a loss in peptide secondary structure. The green circles in the random coil segment of the peptide correspond to hydrophobic residues that interact favorably with PS. The red circles correspond to hydrophilic residues that prefer not to interact with PS.

The pH dependence of the secondary structure of the amphiphilic conjugates was also investigated. The concentration dependence of the percent helicity of 1CW-PS1K in pH 3, 5, 8, and 11 buffers with 0.45 wt% OG is plotted in Figure 4.5. Peptide helicity increases with increasing peptide concentration and reducing pH, as was observed previously for the lone peptide. The decrease in helical content upon PS conjugation to the N-terminus of the peptide is less dramatic as the pH increases. To exemplify, the helical content of 1CW at pH 3 and 5 is ~80%, while that of 1CW-PS1K is ~40%. At pH 8, 1CW is ~65% helical, whereas 1CW-PS1K is ~38% helical. At pH 11, the helical content of 1CW drops to ~45%, while that of 1CW-PS1K is ~35%.

![Figure 4.5. Concentration dependence of the percent helicity of 1CW-PS1K in pH 3, 5, 8 and 11 buffers, with 0.45 wt% OG.](image)

The change in thermal stability of the peptide secondary structure upon polymer conjugation was investigated by taking temperature dependent CD measurements. Figure 4.6 shows the percent helicity for 1CW, 1CW-PEG2K, and 1CW-PS1K upon heating. The peptide-polymer conjugates display significantly higher helical contents than the
peptide itself at high temperature. 1CW displays an obvious melting point at ~70 °C, whereas the PEG and PS conjugates do not display obvious melting points over the range studied. It appears that polymer conjugation to the peptide, regardless of the type of polymer, changes the folding behavior of the peptide and reduces the cooperativity of the folding process. The enhanced thermal stability of the peptide upon PEG conjugation has been observed previously and was attributed to the local microhydrophobic environment provided by the presence of PEG, as well as the PEG chains acting as osmolytes that promote helix formation.\textsuperscript{79-81} A similar thermal stability effect was observed with the same peptide in a previous study where polymers were conjugated to the exterior of coiled-coils.\textsuperscript{73} For 1CW-PS conjugates, DLS results showed that the amphiphilic conjugates form micelles in 0.45 wt% OG solution, thereby protecting the peptide from drastic changes in temperature. This observation is similar to that in a recent study of a noncovalently linked PS-heterodimer-PEG triblock copolymer, which was also attributed to micelle formation.\textsuperscript{50}

![Graph](image.png)

\textbf{Figure 4.6.} CD temperature melts of ~50 μM solutions of 1CW, 1CW-PEG2K, and 1CW-PS1K in pH 8, 0.45 wt% OG buffer.

The second peptide studied, known as H10H24, forms an anti-parallel coiled-coil 4-helix bundle that can bind up to four hemes per bundle via bis-histidyl ligation. Use of this peptide extends studies from a model, \textit{de novo} designed, 3-helix bundle (\textit{i.e.} 1CW) to a naturally-derived, functional, 4-helix bundle, whose heme-binding capability allows for further interrogation of the tertiary structure of the peptide, with a focus on the binding pocket within the interior of the helix bundle. H10H24 is based on the diheme cytochrome \textit{b} subunit of cytochrome \textit{bc1}.\textsuperscript{71,72} The peptide contains two histidines at positions 10 and 24 that serve as heme binding sites and may enable us to address the
extent of helix unwinding induced by the hydrophobic PS conjugation. Such a study will also shine some light on the effects of hydrophobic polymers on the built-in functionalities of coiled-coil helix bundles. Many functional peptide motifs have been *de novo* designed based on H10H24 to either mimic natural redox proteins to convert solar energy to electrochemical potential or to incorporate novel functionalities not seen in nature.\textsuperscript{51-61} It is critical to evaluate the effect of hydrophobic polymer conjugation on cofactor binding and to provide guidance in designing amphiphilic peptide-polymer conjugates toward functional biomolecular materials.

As in the case of 1CW-PS1K, DLS indicates that H10H24-PS1K forms micelles ~8.8 nm in size when solubilized in 0.45 wt% OG, pH 8 buffer, as shown in Figure 4.7. As in the case of 1CW, the OG dependence of the helical content of H10H24 alone was also investigated to decouple surfactant effects. There was minimal effect of OG on the secondary structure of the unmodified peptide at 0.45 wt% OG, but there was a slight decrease in helical content as the OG concentration was increased above this concentration. Figure 4.8 compares the CD spectra of H10H24, H10H24-PEG2K, and H10H24-PS1K in 0.45 wt% OG buffer at pH 8, with all traces showing typical α-helix characteristics. H10H24, the unmodified peptide, displays a helical content of ~80%. The helicity drops to ~70% for H10H24-PEG2K and ~60% for H10H24-PS1K. Similar to the trend observed with 1CW and its conjugates, the CD results further confirm that polymer conjugation to the N-terminus decreases the helical content of the peptide, with a more drastic reduction observed with the hydrophobic PS than with the water-soluble PEG.

![Graph showing DLS of H10H24-PS1K (Dh ~ 8.8 nm) in pH 8 buffer, 0.45 wt% OG.](image)

**Figure 4.7.** DLS of H10H24-PS1K (Dh ~ 8.8 nm) in pH 8 buffer, 0.45 wt% OG.
Figure 4.8. CD spectra of ~50 μM solutions of H10H24, H10H24-PEG2K, and H10H24-PS1K in 25 mM KH$_2$PO$_4$ buffer at pH 8, with 0.45 wt% OG. All traces show typical α-helix characteristics. Inset: Concentration dependence of the percent helicity of H10H24, H10H24-PEG2K, and H10H24-PS1K in 25 mM KH$_2$PO$_4$ buffer at pH 8, with 0.45 wt% OG.

In order for heme to be incorporated into the H10H24 helix bundle via bis-histidyl ligation, the secondary and tertiary structure of H10H24 must remain intact, with the histidine residues from two adjacent helices aligned to preserve the binding site. Partial unfolding of the H10H24 helices would compromise the integrity of the heme binding pocket. In aqueous solution, H10H24 forms anti-parallel 4-helix bundles.$^{71,72}$ Upon attaching an alkyl tail to the N-terminus, amphiphilic H10H24 forms parallel 4-helix bundles at the air-water interface.$^{82}$ Since H10H24-PS conjugates form micellar assemblies in 0.45 wt% OG buffer solution, we expect the topology to be a parallel 4-helix bundle. Thus, the heme binding experiments may potentially elucidate the segment of the peptide that unfolds due to PS conjugation. From CD measurements, H10H24 is ~80% helical, which corresponds to both histidine binding sites falling within the helical structured segment. Therefore, it is expected that each H10H24 helix bundle would bind 4 hemes. Upon PS conjugation to the N-terminus, the peptide unwinds dramatically, and the histidine closest to the N-terminus, H10, no longer falls within the helical segment of the peptide, but rather within the random coil. It is expected that this binding site would be lost, and each bundle of H10H24-PS1K is only expected to bind 2 of the possible 4 hemes. With PEG conjugated to the N-terminus, it was expected that all heme binding sites would remain preserved because the slight loss in helical content of the peptide does not extend to the histidine residues, so their specified positions remain intact.
Figure 4.9. (a) UV-Vis spectra of heme titrations into a ~4 μM solution of H10H24-PS1K recorded in a 1 cm path length quartz cuvette, upon addition of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, and 8 equivalents of heme per 4-helix bundle. The vertical line indicates the peak at 412 nm, which corresponds to the absorbance of heme bound in the interior of the bundle. (b) The absorbance at 412 nm vs. the [heme]/[4-helix bundle] ratio for H10H24, H10H24-PEG2K, and H10H24-PS1K. The ratio at which the slope changes in each data set indicates the number of hemes that each bundle binds.

To verify these speculations and to investigate the effect of polymer end-conjugation on the interior of the coiled-coil, UV-Vis heme titrations of the peptides and their conjugates were carried out. Figure 4.9a shows the UV-Vis spectra of the titration of heme in DMSO into an aqueous solution of 4 μM H10H24-PS1K. Agitation results in facile incorporation of heme into the peptides, as evidenced by an increase in the Soret peak at 412 nm and poorly resolved Qα and Qβ bands at 560 and 529 nm, respectively. The UV-vis absorption spectrum of the holo-form H10H24-PS1K confirmed that heme is incorporated in a hydrophobic environment via bis-histidyl ligation. As more heme was added, a blue-shift in the peak, due to the absorbance of free heme in solution, was observed. Figure 4.9b plots the absorbance at 412 nm as a function of the heme to helix bundle ratio for H10H24, H10H24-PEG2K and H10H24-PS1K. The ratio at which the slope of each data set changes corresponds to the number of hemes that bind to each bundle. This change in slope for conjugated PS occurs at a lower stoichiometry of heme to four-helix bundle than the unconjugated peptide and the PEG conjugate, indicating that there is a substantial deleterious consequence of PS conjugation to heme binding. There appears to be minimal difference in heme binding between H10H24 and H10H24-PEG2K. The fitting of dissociation constants, as was previously described, revealed that both H10H24 and H10H24-PEG2K bind nearly four hemes per bundle, whereas H10H24-PS1K only binds two. Heme titration of H10H24-PS1K at higher concentration, 30 μM, was also performed to provide consistency with the CD studies (Appendix A.3). This showed the same heme binding behavior as that at low concentration, confirming...
that PS conjugation does disrupt the binding pocket and inhibit heme binding. With the loss of secondary structure upon PS conjugation to the N-terminus, there is necessarily an adverse effect on heme binding, as shown schematically in Figure 4.10.

Figure 4.10. Idealized schematic drawing of H10H24-PS1K, a linear diblock peptide-polymer conjugate based on a coiled-coil 4-helix bundle motif, and the effect of PS conjugation to the N-terminus of the helix bundle-forming peptide. As discussed, the conjugation of a hydrophobic polymer to the N-terminus of the peptide leads to a loss in peptide secondary structure and inhibition of heme binding within the interior of the bundle. The green circles in the random coil segment of the peptide correspond to hydrophobic residues that interact favorably with PS. The red circles correspond to hydrophilic residues that prefer not to interact with PS.

The last factor addressed was the distance dependence of this hydrophobic interaction between the peptide and PS. To further discern the effect of hydrophobicity as a function of distance, an extended loop, composed of six amino acids, was inserted between the H10H24 peptide helix and the PS block. We hypothesized that the added loop may increase the distance between the hydrophobic PS block and the coiled-coil and may potentially reduce the peptide/PS interactions at the peptide/polymer interfaces. Details regarding experiments with the addition of the linker are described in the Appendix. However, no increase in the helical content nor recovery of heme binding were observed. Thus, we speculate that with micelle formation, the added loop did not provide sufficient cushion to shield the H10H24 hydrophobic residues at the a and d positions near the N-terminus from interacting with the PS block.

Comparison of the structural and functional integrity of the peptide and the PS conjugate in a fully hydrophobic environment was carried out by blending the peptide and its PS conjugate with a PS homopolymer and casting into thin films. Both films
contained equivalent amounts of peptides. The CD results, shown in Figure 4.11a, indicate that the peptides retain their α-helical character in thin films, and the UV-Vis spectra, shown in Figure 4.11b, indicate that they are still able to bind heme upon solvent annealing of the film. As it is difficult to quantify the peptide concentration in the thin film for exact determination of the helical content of the peptide and the extent of heme binding, only qualitative comparison between H10H24 and H10H24-PS1K blended with PS could be made. Because the H10H24 in PS film displays higher helical content and more heme binding in comparison to the H10H24-PS1K film of equal concentration of peptide, following the same trend observed in solution, it was concluded that the PS thin film environment has little effect on the peptide structure and function. There appears to be a strong distance dependence of the hydrophobic interaction between the peptide and PS, as the peptide itself is still functional and helical when physically blended with PS, but its integrity is greatly impaired when the PS chains are covalently bonded to the peptide.

Figure 4.11. (a) CD spectra of thin film blends of H10H24 and H10H24-PS1K in PS homopolymer, each containing equal amounts of peptide and 1 equivalent of heme, deposited on quartz substrates. Both traces show typical α-helix characteristics. (b) UV-Vis spectra of thin film blends of H10H24 and H10H24-PS1K in PS homopolymer, each containing equal amounts of peptide and 1 equivalent of heme, deposited on quartz substrates. The vertical line indicates the peak at 412 nm, which corresponds to the absorbance of heme bound in helix bundles via bis-histidyl ligation.

§ 4.3 Conclusion

Linear diblock copolymer-type coiled-coil helix-forming peptide-polymer conjugates have been constructed by covalently linking PEG and PS, respectively, to the N-termini of helix bundles. It was shown that conjugated hydrophilic PEG chains slightly decreased helix folding, but retained full cofactor binding capacity within the bundle core. Conjugated hydrophobic PS chains, however, proved to unwind the helices
dramatically and cofactor binding in the interior of the helix bundle was greatly inhibited. This apparent difference is a result of the hydrophobicity of the polymer chains and intermolecular interactions between peptide residues and hydrophobic polymers. The more hydrophobic synthetic polymer has a greater deleterious effect on the peptide secondary structure and bundle functionality, though these effects were only observed when the hydrophobic polymer was conjugated to the peptide, and not when they were merely blended, further suggesting distance-dependent peptide-polymer interactions.

Based on the results presented here coupled to previous studies, the effect of a hydrophobic polymer on a peptide depends greatly on the hydrophobic periodicity of the peptide sequence and reflects various energetic competitions, including intramolecular interactions for a single peptide, intermolecular interactions among peptide helices, and peptide-polymer interactions. The coiled-coil helix bundles considered here consist of amphipathic peptides, and hydrophobic interactions and electrostatic interactions between helices stabilize helix bundle formation and bury hydrophobic amino acids into the core. Upon covalently linking a hydrophobic polymer to the peptide, the spatial arrangements of amino acids at the peptide/polymer interface change to minimize the unfavorable interactions between PS and hydrophilic residues, as well as to maximize favorable interactions with hydrophobic residues. This leads to observations that hydrophobic polymers act to unwind peptides that form coiled-coils, but favor helix folding for peptides that do not form higher order structures. The effects of the surfactant also need be considered. Present studies were performed at 0.45 wt% OG, below the CMC, where there was minimal change to the peptide helicity and oligomeric state. At this concentration, the surfactant merely serves to stabilize micelle formation of the amphiphilic conjugate.

For the peptide-PS conjugates considered here, the unfolding of the peptide helix at the peptide-PS interface essentially modifies the conjugate from a linear diblock copolymer to a tri-block copolymer. The unfolding is expected to change the length, shape, size and cross-sectional area of each block, in addition to the inter-segmental interaction parameter. Amphiphilic peptide-polymer conjugates hold great promise in generating functional, hierarchical nanostructures. Before reaching their full potential, these studies showed that much consideration needs to be paid to polymer-peptide interactions when designing amphiphilic conjugates in order to achieve targeted assemblies while maintaining the functional integrity of the biomolecular building block.

§ 4.4 Experimental section

4.4.1 Materials

Two peptides, referred hereafter as 1CW (Ac-EVEALEKKVAALESKQVQALEKKVEAELHGWDGR-CONH₂) and H10H24 (Ac-GGGEIWKLHEEFLKKFEELLKLHEERLKKM-CONH₂) were investigated and previously described in detail.⁷⁰,⁷¹ H10H24 is based on a sequence that originally had an
extra cysteine on its N-terminus to facilitate disulfide linkage between two helices, so that the two histidines fell at positions 10 and 24, respectively. The terminal cysteine was deleted for our studies. Though the histidines now fall at positions 9 and 23 of our sequence, they will still be referred to as H10 and H24, and the peptide itself as H10H24, for convenience. The peptides were synthesized on a Protein Technologies Prelude solid phase synthesizer using standard 9-fluorenylmethyl carbamate (Fmoc) protection chemistry on Wang resin (Nova Biochem), typically at 0.1 mmol scale. For the synthesis of 1CW-PS1K and H10H24-PS1K conjugates, prior to peptide cleavage from the resin, monocarboxy terminated PS of molecular weight 1000 g/mol (Polymer Source) was coupled to the N-terminus of the peptide for one day. Coupling leads to low yields, as low as ~25%. For the synthesis of 1CW-PEG2K and H10H24-PEG2K, cysteine was re-appended to the N-terminus to facilitate coupling of maleimide-functionalized PEG of molecular weight 2000 g/mol (Rapp Polymere). The peptides were cleaved from the resin and simultaneously deprotected using 90:8:2 trifluoroacetic acid (TFA)/ethanediol/water for 3.5 hr. Crude peptides were precipitated in cold ether and subsequently dissolved in water and lyophilized, resulting in a white powder.

4.4.2 Reversed-phase high-pressure liquid chromatography
Peptides and their conjugates were purified by RP-HPLC (Beckman Coulter) on a C18 column (Vydac). The flow rate was 10 mL/min for semi-preparative runs and peptides were injected at a concentration of 10 mg/mL. Peptide elution was monitored with a diode array detector at wavelengths of 220 nm and 280 nm. Water-soluble conjugates were eluted with a linear AB gradient, where solvent A consisted of water plus 0.1% (v/v) TFA and solvent B consisted of acetonitrile plus 0.1% (v/v) TFA. For 1CW-PEG2K conjugates, a linear gradient of 37 to 42%B over 25 min was used, with typical elution between 40-41%B. H10H24-PEG2K eluted ~39%B on a 30 to 40%B gradient over 30 min. Amphiphilic conjugates were eluted with a linear AB gradient, where solvent A consisted of water plus 0.1% (v/v) TFA and solvent B consisted of 60% isopropanol, 30% acetonitrile, and 10% water plus 0.1% (v/v) TFA. For purification of 1CW-PS1K and H10H24-PS1K, the linear AB gradient of 50 to 100%B over 50 min was used, with typical elution between 85-100%B.

4.4.3 Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
The identity of the peptides was verified by MALDI-TOF mass spectrometry using α-cyano-4-hydroxycinnamic acid matrix. Other matrices were investigated, including 2,5-dihydroxy-benzoic acid, sinapic acid, and dithranol, with less success. Mass spectra were recorded on an Applied BioSystems Voyager-DE Pro.

4.4.4 UV-Vis
Peptide-polymer conjugates were dissolved in buffered aqueous solution containing 25 mM potassium phosphate (KH₂PO₄) at pH 8, 100 mM potassium chloride (KCl), and 0.45 wt% n-Octyl-β-D-Glucopyranoside (OG), unless otherwise stated. OG, a
common nonionic surfactant, was added to solubilize amphiphilic conjugates. To maintain consistency, OG-containing buffers were also used for studies of water-soluble conjugates. UV-Vis spectra were recorded on a Hewlett-Packard 8453 spectrophotometer using a standard 1 cm path length quartz cuvette. Rather than using the absorbance at 280 nm due to each peptide’s lone tryptophan residue for concentration determination, as is typically done, 290 nm was chosen to minimize the contribution from the absorbance of polystyrene at 260 nm. Using samples of pure peptide, the extinction coefficient at 290 nm was determined based on the concentration garnered from the 280 nm absorbance and its extinction coefficient of 5500 M⁻¹cm⁻¹. Peptide concentrations in solution were determined by their absorption at 290 nm, according to the Beer-Lambert Law and using an extinction coefficient of 4900 M⁻¹cm⁻¹.

Titration experiments were done with 1mL of ~4 μM solutions of H10H24 or H10H24-PS1K in quartz cuvettes. UV-Vis spectra were recorded after addition of each 1 μL aliquot of a 200 μM hemin solution in DMSO. The dissociation constants, Kd’s, of heme binding sites were determined by monitoring the shift of the heme absorbance at 412 nm as a function of the ratio of heme to 4-helix bundles and assuming extinction coefficients of 120,000 M⁻¹cm⁻¹ for one bound heme per bundle and 35,000 M⁻¹cm⁻¹ for free heme. Kd was calculated as described previously. Titration experiments were repeated at least five times to ensure reproducibility.

4.4.5 Dynamic light scattering
DLS size measurements were taken on a Malvern Zetasizer Nano-ZS with a 633 nm laser and a scattering angle of 17° to determine the hydrodynamic radius of samples in solution. Samples were passed through 0.22 μm filters prior to the experiment.

4.4.6 Circular dichroism
CD measurements to characterize peptide secondary structure were made on a Jasco J810 spectropolarimeter. CD spectra were collected from 260 to 190 nm at 0.2 nm intervals, a rate of 20 nm/min, a response time of 4 s, and a bandwidth of 1 nm. Each sample was recorded 3 times and averaged. 1 mm path length quartz cuvettes were used for solution samples. To determine the dependence of peptide structure on the concentration of OG, samples were dissolved in 25 mM KH₂PO₄, 100 mM KCl, and varying amounts of OG (0.45 wt%, 0.9 % and 1.2 %) buffer at pH 8. To determine the pH dependence of the peptide and its PS conjugates, samples were dissolved in 25 mM KH₂PO₄, 100 mM KCl at pH 3, 5, 8, and 11, respectively. Ellipticity was reported as described previously. Helical content was taken to be directly proportional to the mean residue ellipticity at 222 nm. The ellipticity of a one hundred percent helical peptide was estimated using the following formula, where n is equal to the number of amino acids:

\[
\theta \, \text{deg} = -40,000 \phi \left(1 - \frac{2.5}{n}\right).
\]

Temperature melt curves were measured using ~50 μM solutions of peptides or peptide-polymer conjugates. The ellipticity was monitored at 222 nm as the temperature increased from 5° to 95°C in 5°C increments at a rate of
1°C/min, with a 1 min equilibration time at each temperature before the measurement was taken.

4.4.7 Thin Film Preparation

H10H24 and H10H24-PS1K were blended with a PS homopolymer and cast into thin films on quartz. PS (Mn=17,000 g/mol) (PDI = 1.09) was purchased from Polymer Source, Inc. (Montreal, Quebec, Canada). A 1 wt% solution of PS was prepared in benzene (270 µL) and combined with 12 mM solutions of peptides or peptide-polymer conjugates in methanol (30 µL), with one equivalent of heme to peptide in 80:20 benzene:DMSO (20 µL) and an additional 80 µL of benzene. The resultant thin films consisted of equal moles of peptides and peptide-polymer conjugates. Thin films were spun cast onto quartz slides for 10 sec at 1000 rpm. Resulting films were approximately 60 nm in thickness, as measured by a Filmetrics F20 interferometer. Thin films were first solvent annealed under water vapor for 60 minutes (100 µL water in a 150 mL sealed container) and then benzene (300 µL) was added to the chamber and annealing continued for 16 hrs.
Chapter 5
Structural Characterization of Micelles based on Amphiphilic Peptide-Polymer Conjugates

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Amphiphiles are able to self-assemble into hierarchical structures, such as micelles, when dissolved in a selective solvent for one of the blocks. It is interesting from a fundamental materials point of view to investigate the self-assembly of biomolecular building blocks in the generation of various hierarchical, functional nanostructures. By controlling their bottom-up assembly, orientation, and segregation, useful materials for both biological and non-biological applications can be rationally designed. The conjugation of hydrophobic moieties to water-soluble peptides and peptide-polymer conjugates facilitates the incorporation of these building blocks into self-assembled structures, such as micelles. The previous chapter explored the conjugation of hydrophobic polymers to peptides. Such studies showed a loss of peptide structure and function upon conjugating short PS chains to the N-termini of helix bundles. As shown, the effects were not merely due to stericity, but to the hydrophobic nature of the polymer, which led to peptide unfolding to maximize favorable enthalpic interactions. Studies done by a colleague in the lab, where hydrophobic polymers were conjugated to the side of helix bundles, also led to the same result. It is important that the engineering of amphiphilicity also not deleteriously affect the inherent structural and functional properties of the peptide. Thus, the use of hydrophobic polymers for the generation of hierarchical, functional nanostructures was ruled out. Rather, the addition of alkyl tails was next explored, as previous studies done in several groups have shown retention of peptide structure upon end-conjugation. In this chapter, it is shown that amphiphiles based on palmitoylated peptide-PEG side-conjugates self-assemble into well-defined, monodisperse spherical micelles ~15 nm in diameter that display tremendous robustness and stability over a range of conditions, making them particularly attractive and suitable for biomedical applications, such as drug delivery, which will be discussed in Chapter 6.

§ 5.1 Design of micelles based on peptide-polymer conjugates

When trying to generate micelles, the key is the proper design of the amphiphile, since the molecular architecture of the amphiphile dictates the specific morphology of the aggregate. The ratio of the cross-sectional areas of the hydrophilic head group and the hydrophobic tail group is given by the geometric packing parameter, which is equal to $V/a_0l_c$, where $V$ is the volume occupied by the tail group, $a_0$ is the cross-sectional area of the head group, and $l_c$ is the length of the tail group, as depicted in Figure 5.1. The value of the packing parameter determines the morphology of the aggregate. When the packing parameter is less than a third, spherical micelles are geometrically favored. With successively increasing values of the packing parameter, cylindrical micelles ($1/3 < V/a_0l_c < 1/2$), vesicles ($1/2 < V/a_0l_c < 1$), and bilayers ($V/a_0l_c = 1$) form.

Design of suitable amphiphiles for the rational generation of spherical micelles can be divided into selection of the hydrophilic head group and the hydrophobic tail group. The peptide-PEG side conjugates are suitable moieties for the head group, as they
are water-soluble, they retain peptide structure, and they provide a large cross-sectional area with the addition of PEG to the exterior of bundles. Next, a suitable tail group must be a hydrophobic moiety appended to the end of the peptide that does not deleteriously affect peptide structure or function. This ruled out the hydrophobic polymer PS studied in Chapter 4. Studies have been done where 2 alkyl tails were appended to collagen-like peptides. These peptide amphiphiles were able to form micelles of the size range of interest, so two alkyl tails, C16, were chosen as the tail group. The packing parameter of the amphiphile was calculated by taking the dimensions garnered by small angle scattering of the head group, discussed in Chapter 3, and the established values for the volume and length of alkyl tails, which are readily tabulated as a function of the number of carbons. The packing parameter was calculated to be 0.235, which is well within in the spherical micelle-forming range.

![Figure 5.1](image_url)

**Figure 5.1.** Schematic of an amphiphile, distinguished by a hydrophilic head group and a hydrophobic tail, which self-assembles to form higher order aggregates when dissolved in a selective solvent. The packing parameter of the amphiphile determines the overall morphology of the aggregate.

§ 5.2 **Structural characterization of micelles**

As shown in Figure 5.2, the designed amphiphile, called 1CW-dC16-PEG2K, consists of a short peptide helix, 1CW, with a polymer chain, PEG2K, covalently attached to the middle of the helix and two hydrophobic tails, C16, attached to the N-terminus with a C6 spacer. The peptides self-associate to form 3-helix bundles with a subunit diameter of ~2 nm, and polymer chains are anchored to the exterior of each helix bundle. As predicted by their estimated packing parameter, these amphiphilic subunits further self-assemble to form spherical micelles, called 3-helix micelles, as schematically shown in Figure 5.2.
Figure 5.2. (a) Schematic drawing of the designed amphiphile containing a short peptide helix with a polymer chain covalently linked to the side chain of the helix and hydrophobic tails attached to the N-terminus; (b) Three amphiphiles associate to form a subunit, where the head group contains a 3-helix bundle with polymers covalently attached to the exterior; (c) Schematic drawing of the micelle formed from self-assembly of the subunit amphiphiles when dissolved in aqueous solution.

The critical micelle concentration (CMC) of the conjugate, which reflects its stability in solution, was first determined using the pyrene encapsulation method. Pyrene is a hydrophobic fluorescent dye, whose fluorescence characteristics change depending upon the polarity of its local environment. Fluorescence excitation spectra were collected, and the ratio between the intensity at 338 and 333 nm is plotted in Figure 5.3a. The point at which the ratio changes dramatically coincides with the CMC, which was determined to be below 4 μM. This is comparable to that of other peptide amphiphile systems. Above the CMC, 1CW-dC16-PEG2K forms uniform micelles, ~15 nm in diameter. Figures 5.3d and 5.3b show the cryo-TEM and negatively stained dried TEM images of micelles. The analytical ultracentrifugation (AUC) results in Figure 5.3c and size exclusion chromatography further confirmed the uniformity of the micellar size. Fitting of the molecular weight of individual micelles from AUC data indicate an aggregation number of ~78 individual amphiphiles, or 26 3-helix bundle subunits.

Small angle scattering was performed to characterize the structure of these micelles in solution. Fitting of the SAXS data, shown in Figure 5.4a, to a spherical form factor confirmed the formation of spherical micelles ~15 nm in diameter, as observed by TEM. The overall polydispersity was determined to be less than 7%, indicating a high degree of monodispersity that is not seen with many other self-assembled systems. SANS was used to characterize the structure of the micelle in more detail because it provides enhanced contrast and the opportunity for contrast variation, as discussed in Chapter 3. Fitting of the SANS curve, shown in Figure 5.4b, confirmed the formation of a spherical
core-shell structure, where the alkyl tails form a hydrophobic core, ~2.8 nm in radius, and the peptide-PEG conjugates form a hydrophilic shell, ~4.6 nm in thickness. These values are consistent with the expected length of the alkyl tails and the peptide-polymer conjugate head groups, respectively. SANS was also performed on amphiphiles with hydrogenated and deuterated alkyl tails solubilized in D₂O. Global fitting of the two data sets confirmed the dimensions garnered for the core and shell, and provided more reliable fits of the system in comparison to fitting of single scattering profiles, since appropriate parameters in the model could be constrained while increasing the number of data points.

Although the overall size and shape of the micelle is well characterized, along with the sizes of the core and the shell, there still remain structural questions to be addressed. The conformation of PEG in the corona is unknown, as it is possible that its conformation in the confined environment of a micelle corona is different from that when associated with the soluble peptide-PEG headgroup (Chapter 3). In order to elucidate the radial distribution of PEG along the micelle corona, the conjugation of deuterated PEG and the subsequent contrast matching of the peptide for SANS measurements are required. Along these lines, such studies may help elucidate the surface characteristics of the micelle, i.e. whether side-conjugated PEG is able to occupy space at the surface of the micelle, thereby providing a stealth layer that masks the charge and interactions of the peptide moiety. Furthermore, it is also of interest to characterize the lateral packing of subunits in the corona to understand their assembly. It may be difficult to deduce such information from SANS measurements, as high signal to noise would be required at high \( q \) to retrieve information at such small length scales. Another option is the use of liquid surface X-ray scattering of Langmuir monolayers of these amphiphiles to model the lateral packing of subunits on the curved surface of the micelle. Characterization of the lateral packing of the subunits is important because the coiled-coil head groups can be used to control the multivalent display of chemical cues. The in-plane correlation length must therefore be determined, as this information can be used to tailor the presentation of ligands in order to garner specific cellular responses. These studies provide fundamental understanding of how various parameters affect the building blocks and their assembly processes, as well as on the structure-property relationship of amphiphilic conjugates, leading to the development of design principles for generating biomolecular functional materials. Further scattering studies on this family of amphiphiles are needed to address these fundamental questions.
Figure 5.3. Physical characterization of nanoparticles. (a) Critical micelle concentration (CMC) is estimated to be ~ 4 μM, as determined by the pyrene encapsulation method. (b) Negatively stained TEM of 1CW-dC16-PEG2K at 1 mg/ml in 25 mM phosphate buffer at pH 7.5. (c) Sedimentation equilibrium analysis of 1CW-dC16-PEG2K at 100 μM in 25 mM phosphate buffer. Fitting of the data (solid line) into a single-species model yields MW of 512 kDa corresponding to 26 trimolecular subunits. (d) Vitreous ice cryogenic TEM of 1CW-dC16-PEG2K at 1 mg/ml in 25 mM phosphate buffer at pH 7.5.
Figure 5.4. (a) Small angle X-ray and (b) neutron scattering of micelles in phosphate buffer at 0.5 wt%. Fitting of the data (solid line) to a core-shell spherical form factor yields a core diameter of ~5.6 nm, a shell thickness of ~4.6 nm, and polydispersity of ~7%.

§ 5.3 Characterization of the stability of micelles

1CW-dC16-PEG2K micelle solutions exhibit exceptional stability against temperature and long-term storage even at high amphiphile concentrations. 1CW-dC16-PEG2K forms micelles spontaneously over a wide range of amphiphile concentrations by simply dissolving the lyophilized amphiphile in aqueous media. Figure 5.5a shows a series of SAXS profiles of 1CW-dC16-PEG2K solutions with concentrations ranging from 0.5 - 16 wt%. Scattering profiles at q > 0.07 Å⁻¹ can be fit to a spherical core-shell model, similar to that shown in Figure 5.4a, confirming the integrity of individual micelles and the absence of random aggregates. As the volume fraction of micelles increases to 34 vol% at 16 wt% of 1CW-dC16-PEG2K, the micelles start to co-assemble into structures with liquid-like ordering reflected by the broad diffraction peak at q ~ 0.035 Å⁻¹ that corresponds to inter-particle distances of ~18 nm. The micelles also exhibit excellent thermal stability. In-situ SAXS profiles of 0.5 wt% and 16 wt% 1CW-dC16-PEG2K solutions, heated from 25°C to 85°C, are shown in Figures 5.5b and 5.5c, respectively. The peptide helicity reduces from 90% - 72% over this temperature range,
indicating that the head group remains approximately helical. At high concentrations, the inter-particle distance decreases during heating, due, more than likely, to an increase in micelle concentration arising from water condensation on the capillary wall during the heating process. The scattering profiles for \( q > 0.07 \text{ Å}^{-1} \) confirms the formation of well-defined micelles even at elevated temperatures. No change in the form factor was observed for the scattering profile of the low concentration sample over the temperature range studied, again indicating that the micelles remain the same size and shape upon heating. The micelles also exhibit exceptional long-term stability at room temperature with no storage requirements: the SAXS profile of a micelle solution remained the same after storage for 5 months at room temperature (Figure 5.5d) and TEM revealed micelle integrity after storage for 9 months at room temperature (Figure 5.5e). The minimal requirements for storage and transport conditions eliminate concerns encountered with other nanocarriers over their practical usage.

Cargo leakage is oftentimes problematic for micellar particles and requires the cargo to be chemically attached to the amphiphile via cleavable linkage. Therefore, Förster resonance energy transfer (FRET) experiments were carried out to access cargo leakage from 1CW-dC16-PEG2K micelles. A lipophilic FRET pair, 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO, donor) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, acceptor), were independently sequestered in 1CW-dC16-PEG2K micelles. Minimal fluorescence due to energy transfer was detected and essentially no cargo leakage was observed after more than 44 hrs of mixing at room temperature, showing an exceptional stability not previously seen in other nanocarriers of this size range (Figure 5.6).

§ 5.4 Importance of peptide tertiary structure on micelle stability

The stability of the 3-helix micelles against variations in temperature, concentration and storage time and the minimal cargo leakage displayed clearly suggest that the energetic barrier to micelle disruption is high and the subunit desorption rate is very low. To delineate the effect of protein structure on micelle assembly and stability, two amphiphile analogs, called “Scmb-dC16-PEG2K” and “Singlehelix-dC16-PEG2K” were also synthesized using a mutated peptide sequence that forms a random coil and a single helix that does not associate into a coiled-coil, respectively. Because the hydrophobic tail groups were kept the same, both analogs have CMCs comparable to that of 1CW-dC16-PEG2K and form micelles of similar sizes. In addition, another analog was developed where the length of the alkyl tails on 1CW-dC16-PEG2K were increased to C18, resulting in 1CW-dC18-PEG2K amphiphiles, to investigate the effect of hydrophobic tail length on micellar structure and stability.
Figure 5.5. Stability of micelles based on 1CW-dC16-PEG2K over a range of conditions.
(a) Concentration dependent SAXS of 1CW-dC16-PEG2K in 25 mM phosphate buffer. Scattering profiles at scattering vector \( q > 0.07 \text{ Å}^{-1} \) can be fit using the micelle form factor for all samples studied. (b) Temperature dependent SAXS of 0.5 wt% solutions of 1CW-dC16-PEG2K in 25 mM phosphate buffer upon heating from 25 °C to 85 °C. (c) Temperature dependent SAXS of 16 wt% solutions of 1CW-dC16-PEG2K in 25 mM phosphate buffer upon heating from 25°C to 85°C. (d) SAXS comparing 0.5 wt% solutions of 1CW-dC16-PEG2K at 0 and 5 months. (e) TEM of 1CW-dC16-PEG2K after storage in solution at room temperature for 9 months.
Small micelles are generally fluid, dynamic assemblies, where the subunit amphiphiles are constantly exchanging with the surrounding media and with other micelles.\textsuperscript{6} The kinetics of subunit exchange of micelles were studied by monitoring the fluorescence recovery of a self-quenching fluorophore, fluorescein, which was attached to the peptide C-terminus.\textsuperscript{7,8} The conjugation of fluorescein was found not to interfere with micelle formation. The dye-labeled micelles exhibit a dramatic decrease in emission intensity on a per fluorophore basis, in comparison to the free dye in solution due to fluorescence self-quenching when the dye molecules are in close proximity. Subunit exchange between labeled micelles and media and between labeled micelles and non-labeled micelles leads to an increase in fluorescence intensity after addition of non-labeled micelles to the solution. Subunit desorption is the rate-limiting step, as there is an energetic penalty for exposing the hydrophobic tail to the solvent. For Scmb-dC16-PEG2K, a significant enhancement of fluorescence intensity (>140\%) was observed over a 4 hr period at 20\(^\circ\)C, as shown in Figure 5.7a. The kinetics of subunit exchange were similar to that of typical PEGylated amphiphiles not based on the coiled-coil. Even faster subunit exchange kinetics and subunit desorption rates were observed for Singlehelix-dC16-PEG2K. However, minimal amphiphile exchange was observed for 3-helix micelles, with an increase in the fluorescence intensity of merely 8\% for 1CW-dC16-PEG2K. Thus, protein tertiary structure, i.e. the formation of 3-helix bundles, plays a key role in micelle stabilization. The self-association of head groups increases the overall molecular weight of the individual subunits and the energetic barrier of exposing the hydrophobic tails to the aqueous solution. Both may slow down the subunit exchange.
Fluorescence self-quenching measurements of 1CW-dC18-PEG2K show only a small change of 4% in fluorescence intensity, indicating improved micelle stability upon lengthening of the alkyl tails. This is as expected, as the longer alkyl tails enhance hydrophobic interactions in the core, which results in an increase in the energetic barrier for subunit desorption.

Micelle formation is driven by the hydrophobic interactions among alkyl tails. Crystallinity in the hydrophobic core also increases the energetic barrier for subunit exchange. To delineate the effect of the hydrophobic core on the subunit exchange kinetics from the effect of coiled-coil formation, differential scanning calorimetry (DSC) was used to measure the melting points of the alkyl tails. DSC curves were measured for 1CW-dC16-PEG2K and amphiphile analogs upon heating from 5°C to 85°C, as shown in Figure 5.7b. All solutions were incubated at 20°C for 16 hrs before the DSC measurements. Scmb-dC16-PEG2K has a sharp endothermic peak with a melting temperature of 42°C; thus, conjugating PEG to the side chain of this random coil did not compromise the ordering and crystallization of the alkyl chain. For Singlehelix-dC16-PEG2K, the melting temperature was reduced to ~33°C. For 1CW-dC16-PEG2K, three broad peaks centered at 17, 25 and 32°C were observed. The endothermic peak centered at 32°C was similar to that of Singlehelix-dC16-PEG2K and should reflect the amphiphiles that have not formed a 3-helix bundle. The cross-section mismatch between the head group and the tail group, especially upon formation of rigid coiled-coils, leads to splaying of alkyl chains. Therefore, the endothermic peaks centered at lower temperatures should correspond to amphiphilies with different oligomeric states. Even though the hydrophobic core was mainly disordered at 20°C (where the self-quenching recovery experiments were carried out), 3-helix micelles exhibit much slower subunit exchange kinetics as compared to micelles with crystalline alkyl cores, i.e. Scmb-dC16-PEG2K. This, again, confirms that the peptide tertiary structures in the corona layer of the micelle provides the driving forces to stabilize micelles. It is worthwhile to note that the energetic contributions from the alkyl chain packing in the hydrophobic core and the various energetic contribution in the coiled-coil peptide-PEG head group are comparable and can be synergistically tailored to modulate the stability of the micelle.

These studies clearly demonstrate the modularity of the system, as each component of this multicomponent system can easily be tailored or engineered to yield desired properties to suit specific applications. The possibilities are numerous, as there are multiple parameters for each component. To name a few, the peptide sequence can easily be redesigned to yield certain structures, functions, or sizes. The chemical nature, size, and position of the polymer can be varied, and lastly, the length and number of alkyl tails can be tuned as desired. The effect of tuning the various properties of the head group and the tail group can result in systematic changes that yield valuable information for both fundamental understanding of the system and for applications development.
Figure 5.7. Effect of protein secondary and tertiary structure on alkyl chain packing and subunit exchange. (a) Time-dependent fluorescence recovery of fluorescein labeled micelles upon the addition of non-labeled micelles. [labeled peptide] = 15 µM; [non-labeled peptide] = 600 µM. Samples were incubated at 20 ºC for 16 hrs before fluorescence measurements. (b) Differential scanning calorimetry (DSC) thermograms probing the molecular packing of alkyl chains in the micelles composed of amphiphiles with different head groups. From top to bottom: Scmb-dC16-PEG2K, Singlehelix-dC16-PEG2K, 1CW-dC16-PEG2K and 1CW-dC18-PEG2K. All samples were incubated for 16 hr incubation at 20 ºC before the DSC measurements.

§ 5.5 Conclusion

The demonstrated strategy, which uniquely combines polymer chains with a common protein structure, i.e. the coiled-coil helix bundle, opens new avenues to generate organic nanoparticles with tunable stability, and thus the time-scale of disassembly. Present studies demonstrate well-defined, monodisperse spherical micelles, ~15 nm in size, that show exceptional stability over a range of conditions. This modular system leads to a versatile approach to generate organic nanoparticles with tailored stability, disassembly, structural morphology, multivalency, and surface functionality to meet specific requirements for nanomedicine. Application of these 3-helix micelles for drug delivery, along with preliminary biological evaluation, will be discussed in the next chapter.
§ 5.6 Experimental section

5.6.1 Peptide sequences

Three peptide sequences were used: “1CW”, “Scmb” and “SingleHelix”.

“1CW” (EVEALEKKVAALECKVQALEKKVEALEHG) is a de novo designed 3-helix bundle peptide. In the current study, 4-5 additional amino acids were appended to the C-terminal without interfering with micelle formation. Results shown for TEM and SAXS were based on the sequence with “WHHH” as the appended sequence. This sequence was selected due to the effective staining for the dry TEM studies. Other studies including AUC, fluorescence self-quenching, and FRET were based on the native sequence of 1CW.

“Scmb” (EGKAGEKAGAALKCGVQELEKGAEAGEGGW) is a control peptide sequence that forms random coil. It was redesigned based on 1CW and has similar characteristics including PI and hydrophobicity.

“SingleHelix” (EAEAAEKKAIAECKAQAEEKAEEAHGW) is a control peptide sequence that forms a single alpha helix. It was designed by mutating valine and leucine at the $a$ and $d$ positions to alanine in order to disrupt the hydrophobic helical bundle interface.

5.6.2 Synthesis

Peptides were synthesized on a Protein Technologies Prelude solid phase synthesizer using standard 9-fluorenylmethyl carbamate (Fmoc) protection chemistry on PEG-PAL resin (Applied Biosystems), typically at 0.05 mmol scale. Fmoc-Lys(Fmoc)-OH (EMD Bioscience) was appended to the N-terminus to allow coupling of two palmitic acid/stearic acid molecules to the N-terminus of the peptide. To modify the C-terminus of the peptide with PEG750 and fluorescein, Fmoc-Lys(Alloc)-OH was coupled at the C-terminus. The Alloc group was selectively removed by utilizing Pd(PPh3)4 catalyst and radical trapping agent PhSiH3 in DCM. The reaction was repeated five times. The resulting free amino groups of lysine were utilized for conjugating carboxy terminated PEG750 and fluorescein using HBTU/DIPEA chemistry. The coupling reaction was performed at room temperature for 24 hours and repeated twice. Peptides were then cleaved from the resin using standard procedures. Cysteine at position 14 facilitates the site-specific coupling of maleimide-functionalized PEG of molecular weight 2000 g/mol to the middle of the peptide sequence.

5.6.3 Cryo transmission electron microscopy

Cryo sample preparation was done on a Vitrobot (FP5350/60). 5 µl of peptide solution were pipetted on a holey carbon grid and blotted for 2 s to remove excess solution. The sample was quickly plunged into liquid ethane and transferred to a cryo holder containing liquid nitrogen. Samples were imaged on a JEOL 4000 microscope at -177 °C using low dose conditions.
5.6.4 **Negatively stained transmission electron microscopy**

Lyophilized peptide powder was dissolved at 1 mg/ml in 25 mM phosphate buffer at pH 7.4. 5 µl of peptide solution was dropped on a discharged holey carbon coated grid (Ted Pella 01824). After removing excess peptide solution, 5 µl of phosphotungstic acid (2 wt%, pH = 3.3) solution was then applied for 2 minutes. Samples were dried in air and examined by a FEI Tecnai 12 transmission electron microscope at 120 kV.

5.6.5 **Small angle X-ray scattering**

SAXS was carried out at beamline 7.3.3 at the Advanced Light Source, Lawrence Berkeley National Laboratory. Samples were dissolved in 25 mM KH$_2$PO$_4$, pH 7.4 buffer at a range of concentrations, from 0.5 wt% to 16 wt%. Samples of the lowest concentration were measured in a homemade circulating flow cell with 0.025 mm thick muscovite mica windows and counted for 5 s 50 times to garner the form factor. Samples of higher concentration were measured in 2 mm boron-rich thin-walled capillary tubes to investigate both the form and structure factors. In-situ temperature studies were performed using a capillary holder connected to a peltier device. Samples were heated from 25°C to 85°C at a ramp rate of 1°C/min and held for 1 min to ensure equilibrium before acquisition of 10 images of 5 s exposures. The sample to detector distance was ~1.7 m, providing a q range of 0.01 to 0.3 Å$^{-1}$, where $q = 4\pi \sin(\theta/2)/\lambda$, $\theta$ = scattering angle, and $\lambda = 1.24$ Å. The X-ray energy was 10 keV. Scattering was collected with a PILATUS detector. 2D diffraction patterns were radially integrated to garner a 1D profile of the scattering intensity. Form factors were fit using the core-shell sphere model included in the SANS software analysis package provided by National Center for Neutron Research at National Institute of Standards and Technology (NCNR-NIST).

5.6.6 **Small angle neutron scattering**

SANS experiments were conducted on micelles with hydrogenated and deuterated alkyl tails at CG-3 at HFIR, Oakridge National Laboratory. 1 m and 7 m configurations were used for all samples, providing a $q$ range of 0.01 to 0.4 Å, with a variable neutron wavelength resolution $\Delta\lambda/\lambda \sim 10\%$. Samples were dissolved in pH 7.4 25 mM KH$_2$PO$_4$, in D2O for enhanced contrast between the solvent and the conjugates. All samples were prepared at a concentration of 5 mg/ml and measured in 1 mm pathlength cuvettes. Samples were counted for 60 min.

5.6.7 **Analytical ultracentrifugation**

Sedimentation equilibrium experiments were performed on a Beckman Optima XL-A at 25 °C with samples solubilized in 25 mM phosphate at pH 7.4. The path length of the cells was 1.2 cm and the An-60Ti rotor was used. Measurements at 5000, 7000, and 10000 rpm were acquired after 10 h of spinning at each speed to ensure equilibrium, which was verified by matching the early and late data sets. The radial distribution of absorbance was monitored at 280 nm. Sample concentrations were 100 µM, and sample...
volumes were 120 µl. The specific volume of 1CW-dC16-PEG2K was estimated to be 0.877 ml/g using the software Sednterp (http://www.jphilo.mailway.com) and relying on the fit of the SANS profile of 1CW-dC16-P2K to a core-shell model with interfacial widths to estimate the number of water molecules that penetrate the shell of the micelle. The density of the buffer was 1.004 g/ml. Nonlinear global fits were made using the UltraScan software program (http://www.ultrascan.uthscsa.edu/).

5.6.8 Differential scanning calorimetry
DSC was performed on a VP-MicroCal calorimeter (GE). ~600 µl of sample and buffer were loaded into two parallel stainless steel cells that were sealed tightly under the pressure of ~27 psi to prevent water evaporation during the heating cycle. The temperature was increased from 5 ° to 85 °C at a rate of 1 °C/min, with a 15 min equilibration time at 5 °. DSC thermograms were obtained after concentration normalization and baseline correction using the Origin software provided by MicroCal.

5.6.9 Dynamics of subunit exchange via self-quenching
Fluorescein-labeled micelles (donor) were prepared at a concentration of 16 µM in 25 mM phosphate buffer at pH 7.4. Non-labeled micelles (acceptor) were prepared at a concentration of ~3.6 mM using the same buffer. The two solutions were mixed in a 5:1 volume ratio, giving a donor:acceptor molar ratio of 1:40. Time dependent fluorescence intensity was recorded every 30 seconds after mixing, with the excitation wavelength of 494 nm and emission at 527 nm.

5.6.10 Förster resonance energy transfer
A lipophilic FRET pair, 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO, donor) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, acceptor) were used to measure the energy transfer upon mixing. DiO and DiI were dissolved in acetone to a concentration at 0.1 mg/ml, respectively. 50 µl DiO and 50 µl DiI were independently added to 0.5 ml of peptide aqueous solution (1 mg/ml, pH=7.4). After 24 hours stirring at room temperature, acetone was evaporated with vials left open for 24 hours. The solutions were then subject to centrifugation and spin dialysis to remove any insoluble aggregates and soluble dyes in the supernatant. The resulting dye-encapsulated nanoparticles were characterized by size exclusion chromatography. Encapsulation of dye molecules within nanoparticles were confirmed by the overlap of elution profiles monitored at 220 nm and 490 nm, respectively for DiO, at 220 nm and 560 nm, respectively for DiI. Time dependent fluorescence intensity was recorded for 44 hours upon mixing the nanoparticle solutions with excitation wavelength at 488 nm.
Chapter 6
Behavior of Amphiphilic Peptide-Polymer Conjugates at the Air/Water Interface

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The previous chapter described the structural characterization of micelles formed from the self-assembly of amphiphilic coiled-coil peptide-polymer conjugates in solution. Here, the 2D phase behavior and properties of the amphiphiles at the air/water interface were investigated. The Langmuir monolayer is a 2D model system that may be correlated to the packing of amphiphiles not only at polar/nonpolar interfaces, but also on the curved surface of micelles. Liquid surface scattering allows for the characterization of subunit packing at an interface, which may elucidate the role of peptide structure in enhancing stability and slowing subunit exchange kinetics of the micelles. It is also important to determine the lateral packing of subunits, as they can be used to control the multivalent display of chemical cues. Investigation of their 2-D phase behavior and the principles governing their assembly will provide guidance for generating unique assemblies for biomedical applications. Furthermore, these studies shed light on our fundamental understanding of the phase behavior of hybrid biomaterials based on peptides and synthetic polymers.

§ 6.1 Introduction

Engineering amphiphilicity into peptide-polymer conjugates is important for achieving many targeted applications. Amphiphilicity may enable them to self-assemble into functional, hierarchical nanostructures.1-8 Like other amphiphilic molecules, such as lipids and synthetic block copolymers, amphiphilic peptide-polymer conjugates can self-assemble into various structures, such as spherical micelles, cylindrical micelles, and vesicles, when dissolved in a selective solvent for one block. In particular, these amphiphiles can be used to generate nanoparticles that hold great potential as nanomedicines.9,10 Effective utilization of such nanoparticles hinges on a few requirements, including well-defined particle size and shape, high cargo loading, enhanced stability, and a controlled disassembly process.11,12 Lipidated coiled-coil-PEG side conjugates form monodisperse, well-defined spherical micelles ~15 nm in diameter that display exceptional stability and robustness over a range of conditions, including temperature, concentration, and time. Because micelles are generally fluid, dynamic species whose subunits constantly exchange with the surrounding media and with other micelles, their stability and cargo leakage are usually limiting factors for many applications, particularly drug delivery. However, the peptide-polymer side-conjugate-based micelles display slow kinetics of subunit exchange and minimal cargo release, making them especially suitable for biomedical applications. They also demonstrate promising behavior in vivo, with a blood circulation half-life-time of ~29.5 hr in mice. The behavior demonstrated both in vitro and in vivo indicate that this system holds tremendous promise for nanomedicine. Micelles based on these coiled-coil-PEG side conjugates are also unique because the tertiary structure of the peptide in the head group is key to the stability of the micelle and offers the ability to tune the multivalent display
of chemical cues for eliciting specific cellular responses. Along these lines, it is important to gain a fundamental understanding of subunit packing.

Furthermore, amphiphilicity provides great flexibility by allowing the peptide to interface with other synthetic polymers, organic solvents, and lipids. In addition to the formation of varied structures in solution, amphiphilicity raises the possibility for incorporation into polymer thin films or at polar/nonpolar interfaces. The Langmuir monolayer is a 2D model system that may elucidate the phase behavior amphiphilic peptide-polymer conjugates at the air-water interface and may subsequently be correlated to the packing of amphiphiles not only at polar/nonpolar interfaces, but also on the curved surface of micelles. Liquid surface scattering allows for the characterization of subunit packing at an interface, which may elucidate the role of peptide structure in enhancing stability and slowing subunit exchange kinetics. It is also important to determine whether the amphiphiles still form coiled-coils at such interfaces. Investigation of their 2-D phase behavior and the principles governing their assembly will provide guidance for generating unique assemblies for biomedical applications. Furthermore, these studies may shed light on our fundamental understanding of the phase behavior of hybrid biomaterials based on peptides and synthetic polymers.

Langmuir monolayers of amphiphilic 4-helix bundle peptides have been well studied in the past. Amphilicity was incorporated either by the addition of lipid tails to the end of the bundle, similar to our system, or by designing hydrophobic residues on the exterior of the bundle, thereby creating a hydrophilic domain on one end of the bundle and a hydrophobic domain on the other. The hydrophobic domain is based on a synthetic ion channel and the hydrophilic domain has designed cavities for binding the general anesthetic halothane. Grazing incidence X-ray diffraction showed that they are able to form 4-helix bundles that are vectorially oriented within Langmuir monolayers at the air/water interface. X-ray reflectivity allowed for the characterization of halothane binding within the hydrophilic domain. Other 4-helix bundles have also been designed to bind extended conjugated chromophores that exhibit large optical polarizabilities and hyperpolarizabilities. The bundles’ designed amphiphilicity enabled vectorial orientation of the chromophore/peptide complex in macroscopic monolayer ensembles. These studies have shown that Langmuir monolayers of amphiphilic helix bundles can be used to generate hierarchical functional nanostructures that display liquid-like ordering at the air/water interface.

Here, X-ray reflectivity was used to study the macroscopic orientation of 3-helix bundle amphiphiles as a function of surface pressure and surface area occupied by each amphiphile. Grazing incidence diffraction was performed to investigate the 2-D lateral packing of the amphiphiles. These studies provide valuable information on the intra- and inter-molecular interactions between the amphiphiles and their effects on the phase behavior of the amphiphiles on a flat surface. They also provided critical information needed to understand the lateral packing of amphiphilic subunits on a curved surface, e.g. micelles. In particular, these studies may help determine the parameters underpinning the unusually high stability of the micelles observed in solution. Such stability is critical for
in vivo studies and validates the usage of these micelles as nanocarriers. Characterization of the lateral packing of the subunits is also important as the coiled-coil head groups can be used to control the multivalent display of chemical cues. The in-plane correlation length must therefore be determined, as this information can be used to tailor the presentation of ligands in order to garner specific cellular responses. These studies provide fundamental understanding of how various parameters affect the self-assembly of the building blocks, as well as on their structure-property relationships, thereby leading to the development of design principles for generating biomolecular functional materials.

§ 6.2 Results and discussion

The amphiphile of interest, 1CW-dC16-PEG2K, was described in detail in the previous chapter. A schematic drawing of the 3-helix bundle-forming amphiphile is shown in Figure 6.1.

![Figure 6.1. Schematic drawing of 1CW-dC16-PEG2K, which self-assembles to form 3-helix bundles.](image)

Circular dichroism (CD) measurements were made to characterize the secondary structure of 1CW-dC16-PEG2K. The CD spectrum displays typical alpha-helix characteristics, with minima at 222 nm and 208 nm and a maximum around 195 nm, as shown in Figure 6.2. The helical content of the peptide is ~75%.30
The pressure-area isotherms of Langmuir monolayers of 1CW-dC16-PEG2K on an aqueous subphase at 25°C are shown in Figure 6.3. It is evident that the amphiphiles form a stable film that assembles into two different organizational states. The initial liquid expanded phase at low surface pressures, in which the long axes of the peptides are parallel to the surface, transitions to a liquid condensed phase, in which the peptides are now perpendicular to the surface at high pressures. This transition is characterized by a steep rise of the isotherm upon compression for areas/helix below 200 Å². The relatively high surface pressure achieved at the minimal area/helix is indicative of the amphiphilicity of 1CW-dC16-P2K. The minimal area of 100 Å²/helix is the estimated cross-sectional area for an individual un-PEGylated, ideally perfectly, straight α-helix oriented with its long axis perpendicular to the interface. For areas/helix above 550 Å², the surface pressure decays to zero. The peptide is expected to be oriented parallel to the interface, with the hydrophobic residues and the alkyl tails exposed to air and the hydrophilic residues and PEG submerged in the subphase. The transition between the two orientations occurs in the plateau region at a surface pressure ~10 mN/m. The isotherm exhibits a pronounced hysteresis between compression and expansion.
As the diameter of an α-helix is about 10 Å, and its pitch is about 1.5 Å/residue, a 30 residue α-helix lying on its side should cover roughly 450 Å$^2$, while its cross-sectional area should be about 100 Å$^2$. When the peptide trimerizes, the diameter of the bundle is expected to be $\sim$18 Å (from known crystal structures). These estimations neglect the presence of alkyl tails and the polymer chain, both of which increase the mean molecular area for each orientation. With consideration of the PEG and alkyl tails, the diameter of the bundle is expected to increase to 26 Å (from small angle x-ray scattering of the head group discussed in Chapter 3) and the length of the peptide with alkane tails is expected to be $\sim$67 Å. Therefore, a helix-bundle lying on its side, assuming that its tails are in air, should cover roughly 390 Å$^2$/helix and its cross-sectional area is 225 Å$^2$/helix. Comparing these numbers with the isotherms suggests that the peptide is oriented with its long axis parallel to the interface at low pressures, until it becomes close packed. Then the molecular axis changes its orientation to normal to the interface, and the surface pressure increases again as the upright helices approach close packing. In this orientation, the α-helices would be in the subphase, while the hydrocarbon chains remain in the air. It is difficult to characterize the formation of coiled-coils and their oligomeric state at the air-water interface from Langmuir isotherms. X-ray reflectivity was performed to observe the behavior of the monolayer directly.

The behavior of the conjugates at the air-water interface was further investigated by X-ray reflectivity using a liquid-surface spectrometer. Reflectivity was taken at
different pressures for each sample: one at low pressure (10 mN/m), one at an intermediate pressure (20 mN/m), and two at relatively high pressures (30 and 40 mN/m). The corresponding Fresnel normalized reflectivity at each surface pressure is shown in Figure 6.4a. Reflectivity was measured at a pressure around the plateau in the isotherm and three at pressures above the plateau, which corresponds to the transition from a liquid expanded to liquid compressed phase. At the lowest pressure of 10 mN/m, the data consist of a single, broad maximum. With increasing surface pressure, the maximum narrows and shifts to smaller \( q_z \), developing subsidiary maxima and minima with an amplitude that decays as \( q_z \) increases. The decrease in periodicity of the oscillations in \( q \)-space with increasing surface pressure corresponds to a dramatic increase in the thickness of the monolayer in real space.

Figure 6.4. (left) Fresnel-normalized reflectivity and (right) the corresponding electron density profiles of Langmuir monolayers of 1CW-dC16-PEG2K at various surface pressures upon compression. Electron density profiles were fit to a two slab model.
The normalized reflectivity was analyzed via the box-refinement procedure to provide, with no a priori assumptions, the electron density profile of the monolayer. Figure 6.4b shows the electron density profiles of 1CW-dC16-PEG2K at various surface pressures. Each electron density profile was fit to one slab and two slab models, with better fits generally obtained with two slabs. The fit parameters are listed in Table 6.1. When fit to a single slab model of constant electron density, the thickness of the monolayer is observed to increase from ~11 Å to ~62 Å when the surface pressure increases from 10 mN/m to 40 mN/m. The orientation of the conjugates in the Langmuir monolayer was thus determined from the electron density profile normal to the interface.

The resultant electron density profiles follow along expected lines, with the long axis of the bundles parallel to the liquid surface at low pressures, and perpendicular to the surface at high pressures.

When fitting the low pressure data to a two slab model, two slabs of lengths 10 Å and 17 Å were best fit to the electron density profile, with the shorter slab at the air-water interface and the longer slab below that. The 10 Å box coincides with the width of single α-helices, whereas the 17 Å-thick slab corresponds to extra electron density due to peptides that have oligomerized to form three helix bundles, with the length of the bundle parallel to the air-water interface. The width of the PEGylated bundle is expected to be ~2.6 nm (from SAS measurements), which agrees well with the total thickness of the two blocks. Therefore, at the low surface pressure of 10 mN/m, the monolayer is composed of a mixture of single peptide amphiphiles and those that have oligomerized to form three helix bundles. For both species, whether monomers or trimers, the peptides are oriented parallel to the interface. The electron density profile of the monolayer at 20 mN/m becomes more defined away from the interface, indicative of more amphiphiles that are oriented normal to the interface. This is expected as 20 mN/m lies above the plateau where the liquid expanded phase transitions to the liquid compressed phase. However, the electron density peak at the interface indicates that a majority of the amphiphiles are still lying down. At 30 mN/m, the thickness of the monolayer increases to ~62 Å, which corresponds to the length of the amphiphile, indicating that they are oriented normal to the interface. The normalized reflectivity at 30 mN/m contains more well-defined oscillations indicative of a more uniform monolayer. Fitting of the electron density profile to a two box model shows an 29 Å thick box near the interface with an electron density 1.09 times (0.36 e/Å³) that of water (0.33 e/Å³) and a 33 Å thick box below that with an electron density 1.10 times (0.37 e/Å³) that of water. The electron density of the alkyl tails and the peptide-PEG head group were calculated to be 0.29 e/Å³ and 0.41 e/Å³, respectively, or 1.24 and 0.88 times that of water, respectively. The lower electron density near the interface corresponds to the presence of alkyl tails at the surface, whereas the more electron dense peptide-PEG head groups are further in the subphase. At 40 mN/m, the electron density profile is similar to that at 30 mN/m, though the monolayer may be rougher due to buckling from the high compression ratio.
Figure 6.5a and 6.5b show the normalized reflectivity and resultant electron density profiles of the monolayer upon expansion. Upon expansion of the monolayer to 30 mN/m, the monolayer relaxes slightly and forms a more uniform film in comparison to the monolayer at 40 mN/m, as evidenced by the sharper features in the normalized reflectivity. Upon further expansion of the monolayer to 20 mN/m, the electron density peak at the interface reappears, indicative of amphiphiles that are laying down. Though the electron density profiles of the monolayer at 20 mN/m are similar upon compression and expansion, the normalized reflectivity upon expansion contains more well-defined features, indicative of a smoother monolayer. This is expected, as the mean molecular area at expansion to 20 mN/m is smaller than that upon compression, as depicted in the isotherm in Figure 6.3.

The data clearly demonstrate that 1CW-dC16-PEG2K undergoes an orientational transition within the monolayer, in which its long axis changes from parallel to the plane of the air-water interface at low pressures to being normal to the interface at high pressures. The electron density profile of the monolayer at low pressure agrees well with the projection of a mixture of single α-helices and 3-helix bundles with their long axes lying in the plane of the interface. By de novo design of 1CW, the hydrophilic residues occur on one face of the helix, while the hydrophobic residues occur on the other. In solution, 3-helix bundles form in order to minimize the free energy of the peptide by
burying all the hydrophobic residues in the interior of the bundle. However, at the air-water interface, the peptide is able to minimize its free energy by exposing its hydrophobic face to the air and its hydrophilic face to the subphase, given enough surface area. The peptides begin to trimerize when the monolayer is compressed such that there is not enough available area for each amphiphile to lie down at the interface.

**Table 6.1**

<table>
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<th>$\pi$ (mN/m)</th>
<th>A/helix (Å$^2$)</th>
<th>$L_1$ (Å)</th>
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<td>20, expansion</td>
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Similarly, the high pressure data of 1CW-dC16-P2K agree well with the expectations for peptide amphiphiles oriented normal to the air/water interface. The total length of the two slab model corresponds to the total length expected of the amphiphile, with a shorter, less dense slab representing the alkyl tails, and the longer, denser slab representing the peptide-PEG head group. The interface between the alkyl tails and the peptide and the interface between the peptide and the subphase are both well-defined, based on the fits. The largest roughness is seen at the interface between the subphase and the alkyl tails.

Lastly, the in-plane lateral organization of amphiphiles was investigated by GID. At high pressures of 30 and 40 mN/m, where the helices are oriented approximately perpendicular to the interface, Figure 6.6 shows that monolayers of 1CW-dC16-P2K produce a single, broad, GID peaks, modeled as Gaussians centered at $q = 0.44$ Å$^{-1}$ (d-spacing ~ 14 Å) and $q = 0.48$ Å$^{-1}$ (d-spacing ~ 13 Å), respectively. Such peaks are absent from GID of the aqueous subphase itself and of Langmuir monolayers of the phospholipid, DPPC. This diffraction arises from the interference between parallel helices, as the dimension corresponds well to the diameter of individual PEGylated $\alpha$-helices, rather than entire helix bundles. This length corresponds to the distance of closest approach between nearest neighbors, and indicates some 2D order of the peptides in the monolayer.
Figure 6.5. (left) GIXD patterns of Langmuir monolayers of 1CW-dC16-PEG2K at various surface pressures. (right) $q_{xy}$ dependence of the GIXD data.

Though it has been demonstrated that these amphiphilic peptide-polymer conjugates are able to assemble at the air-water interface, greater understanding of the behavior and monolayer properties is necessary. Future publications will compare this model amphiphilic peptide-PEG conjugate, 1CW-dC16-P2K, with analog amphiphiles whose components have been systematically varied. With this modular system, the proposed future studies will elucidate the role of each component on monolayer
formation and subunit packing within the monolayer. Such fundamental studies for extracting the 2D phase behavior of the amphiphiles are required in order rationally design functional, self-assembled nanostructures with applications in biomedicine and beyond.

§ 6.3 Conclusion

The behavior and properties of Langmuir monolayers of amphiphilic 3-helix bundle-forming peptide-polymer conjugates were investigated. By performing reflectivity and grazing incidence diffraction on Langmuir monolayers of amphiphilic peptide-polymer conjugates, we aim to gain an understanding of their phase behavior at the air-water interface. Reflectivity provides the vertical orientation and grazing incidence diffraction helps to determine and the in-plane lateral organization of the amphiphiles as a function of surface pressure.

We have demonstrated that 1CW-dC16-PEG2K forms stable monolayers with the bundles oriented normal to the air-water interface with some lateral order. Subsequent studies can focus on the systematic variation of each component of the modular system. The position and molecular weight of the polymer along the length of the helix, the number and length of alkyl tails, and the intra- and intermolecular interactions of the peptide can all be varied to determine the effects of each component on the vertical orientation and the lateral packing of the subunits. These studies may lead to an understanding of the phase behavior of the amphiphiles at the air/water interface and of the parameters necessary for the stability of micelles in solution.

Surface pressure-area isotherms indicate a transition from a liquid expanded state at low pressures, where bundles are oriented parallel to the air-water interface, to a liquid compressed state at high pressures, where bundles are oriented normal to the interface. The electron density profiles of the conjugates normal to the interface, which were extracted from X-ray reflectivity measurements via a model-independent box refinement procedure, confirmed the phase transition upon compression and the formation of a stable, uniform monolayer. Grazing incidence diffraction, which was conducted in order to garner information about the in-plane, lateral organization, indicates the presence of some order of upright helices.

§ 6.4 Experimental section

6.4.1 Langmuir monolayer

Isotherms were collected using a KSV 2000 Langmuir trough on a vibration isolation table. The spreading solution was typically a ~100 μM concentration of
amphiphiles dissolved in methanol and the subphase is 1 mM phosphate buffer with 10 mM KCl at pH 7.4. To promote the formation of a monolayer, a microliter pipet was used to deposit the solution dropwise at the thin film of water provided by the meniscus that forms over a glass capillary penetrating the air-water interface at an oblique angle. The monolayer was allowed to equilibrate for 20 min before compression. Compression was carried out at 10 mm/min. Surface pressure was monitored using a Wilhelmy plate.

### 6.4.2 Liquid surface spectrometer

We performed all X-ray scattering experiments at ChemMatCARS Sector 15 of the Advanced Photon Source (Argonne National Laboratory) on a liquid surface spectrometer. Description of the liquid surface spectrometer and the configurations for X-ray reflectivity (XR) and grazing incidence diffraction (GID) can be found in detail elsewhere. For XR measurements, the spectrometer was kept in the reflectivity condition, where $\alpha = \beta$, to collect the photons scattered with momentum transfer perpendicular to the liquid surface ($q_z = 4\pi \sin(\alpha)/\lambda$). Reflectivity was collected between $0.018 < q_z < 0.6 \text{ Å}^{-1}$. GID was carried out to garner in-plane electron density distribution profiles of the monolayer. The incident angle was set such that $q_z = 0.016 \text{ Å}^{-1}$, below the critical angle of water ($q_z = 0.0217 \text{ Å}^{-1}$). GID was scanned in the region $0.5^\circ < 2\theta_{xy} < 19^\circ$ to determine the presence of any order of alkyl chains, $\alpha$-helices, or helix bundles, depending on the $q$-range of interest.

After deposition of the monolayer as described above, the monolayer was allowed to equilibrate for 20 min. Compression was carried out at 10 mm$^2$/s until the system reached its desired target pressure, and then constant pressure was maintained during the X-ray measurements. The trough was translated transverse to the beam by 1 mm to expose a fresh portion of the sample to the beam after each reflectivity scan and thereby avoid any potential radiation damage to the peptide monolayer.

### 6.4.3 Data reduction and analysis

We performed XR to obtain out-of-plane and electron density distribution. Analysis can provide information about the composition across the interface with angstrom-level resolution. The high brilliance of the XR beam at the APS provides high-quality XR data up to 0.6 Å$^{-1}$. StochFit, which is a modeling approach developed at the University of Chicago whereby a stochastic Monte Carlo algorithm is used to search through the solution space of all possible electron density profiles, was used to determine the electron density profiles of monolayers with no a priori assumptions. The resultant electron density profiles were modeled with boxes of constant electron density and thickness. The interfaces between boxes are smoothed with a Gaussian function of standard deviation $\sigma$ to account for roughness at the boundary due to thermally excited capillary waves and the atomic roughness of the interface.
Chapter 7

Biological Evaluation of Micelles based on Amphiphilic Peptide-Polymer Conjugates

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Stable, multi-functional organic nanoparticles that combine long in vivo circulation, the ability to cross vessel walls to reach tumor tissues and controlled disassembly/degradation for eventual clearance will have a significant impact in nanomedicine. Although current self-assemblies of amphiphiles provide a versatile platform to generate modular organic nanoparticles, it remains a significant challenge to simultaneously control the nanoparticle size in the range of 10-30 nm, enhance particle stability and tailor disassembly at the timescale suitable for nanocarriers. Towards these ends, the 3-helix micelles described in the previous chapter display many desirable properties for such nanomedicine applications. These include the optimal size range for long circulation times and deep tumor penetration, enhanced stability and long-term shelf-life, slow kinetics of subunit exchange, and minimal cargo leakage. This chapter investigates the biological application of these materials at the in vitro cell level and the in vivo animal model level. As will be described, the resultant 3-helix micelles demonstrate great potential as drug delivery vehicles, as they display long blood circulation half-life times, reduced accumulation in the liver and spleen, and the ability to deliver cargo to cells.

§ 7.1 Need for drug delivery vehicles in cancer therapy

There is a clear need for drug delivery systems to improve the efficacy of drugs. It has been estimated that \( \sim 40\% \) of emerging small molecule drugs have poor aqueous solubility and a short circulation half-life\(^1,2\) and require the development of effective drug formulations to improve their pharmacokinetics, biodistribution, toxicity profile and efficacy.\(^3-12\) Towards these ends, nanoscopic organic particles are attractive as nanocarriers as well as targeting and reporting devices.\(^7,9,12-17\) Nanoparticle-based cancer therapeutics are of particular interest since the carriers reduce the systemic toxicity of chemotherapeutics and are concentrated within tumors via the enhanced permeation and retention (EPR) effect, defined by leaky vasculature and poor lymphatic drainage commonly seen in solid tumors,\(^18-22\) or endothelial transcytosis.\(^23,24\) Studies have shown that following extravasation into tumor interstitium, a drug or drug-encapsulated vehicle should be capable of transport up to 100 \( \mu \)m away from the tumor vasculature in order to reach all cells within the tumor.\(^25\) There is increasing evidence that a drug’s limited penetration and distribution within a tumor, which results in insufficient elimination of malignant cells, may contribute to tumor re-population after treatment.\(^26-29\)

§ 7.2 Overcoming physiological barriers: requirements of nanocarriers

Systemic delivery of therapeutics is a three-step process, defined first by blood-borne delivery to different regions of the tumor, second by transport across vessel walls,
and third by passage through the interstitial space to reach the tumor cells. Each of these steps is largely dependent on the size of the therapeutic, as depicted in the left schematic of Figure 7.1. For long blood circulation half lives, particles must be larger than 10 nm to avoid extravasation in nontarget tissue and renal excretion. At the same time, they must be smaller than 200 nm to avoid opsonization and clearance via the reticuloendothelial system (RES). For crossing tumor vessel walls via the EPR effect, the optimal size of nanomedicines is a couple tens of nanometers. Physiological factors, including the density and heterogeneity of the vasculature at the tumor site, interstitial fluid pressure, and transport of carriers in the tumor interstitium, impact the extent of extravasation of nanocarriers into tumors. Furthermore, nanocarriers need to be below a certain size to achieve significant penetration, and the range of nanocarrier diameters for efficient tumor penetration depends on the shape, hardness and architecture of the carrier. Recent studies using a human melanoma xenograft model in mice showed that smaller particles, i.e. 10-12 nm quantum dots, can more effectively penetrate the physiological barriers imposed by abnormal tumor vasculature and dense interstitial matrix than 60 nm and 125 nm nanoparticles. Real-time intravital imaging showed that the 12nm particles extravasate easily and diffuse away from vessels with minimal hindrance, whereas 60 nm nanoparticles extravasate but do not leave the immediate perivascular space, and the 125 nm particles barely extravasate at all. Using dendrimers, the physiologic upper limit of pore size in the blood-tumor barrier of malignant solid tumor microvasculature is approximately 12 nm. Organic nanoparticles based on elastin-like peptides, ~25 nm in size, produced a nearly complete tumor regression in a murine cancer model. Thus nanoparticles in the range of 10-30 nm with long circulation half-lives and the ability to penetrate into tumors are highly desirable to deliver chemotherapeutic drugs.

Effective carriers require extended blood stability and the ability to extravasate, diffuse through the interstitial space and cross tumor cell membranes. Further, to engineer practical therapeutics, the nanoparticles should have a long-term shelf life without refrigeration. For reduced toxicity, rapid renal clearance of the nanoparticle components is also critically important. Ideally, the nanocarriers should disassemble or degrade into non-toxic subunits less than 5 nm for eventual renal clearance. Few, if any, current drug delivery formulations combine a plasma half-life of tens of hours with eventual sub-unit renal clearance.

The effectiveness of a drug carrier also depends on its stability and drug retention in vivo. To ensure an improvement in the toxicity profile of the drug, the drug needs to be retained within micelles until reaching the target site. In addition to enhanced cargo stability and tumor penetration, an equally important requirement for effective nanocarriers is the balance of stable circulation and nanocarrier clearance. Nanocarriers initially must be larger than 6 nm to achieve extended circulation lifetimes and subsequently need to disintegrate into materials smaller than ~6 nm or 50 kDa in molecular weight to be eliminated from circulation by glomerular filtration in the kidneys. The generation of organic nanocarriers in the size range of 10-30 nm which
combine a long circulation half-life, effective tumor tissue penetration, minimal cargo leakage, and efficient subunit clearance remains a significant challenge.

**Figure 7.1.** Schematics comparing the length scales of various physiological barriers of systemic delivery of therapeutics (left) and various formulations of drug delivery vehicles that have been developed and investigated (right). The red target on the right schematic symbolizes what we consider to be the optimal size of nanocarriers.

§ 7.3 **Existing nanomedicines**

There exist a host of formulations designed for drug delivery, including polymer drug conjugates, polymeric micelles, nanospheres, vesicles, and dendrimers, as depicted in the right schematic in Figure 7.1b. At the bottom of the figure are the size ranges of the various technologies, with polymer drug conjugates and dendrimers usually below 10 nm and nanospheres and vesicles above 100 nm. Micelles span the range in between, from 10 to 100 nm, which is the optimal size for cancer drug delivery, as discussed above. Thus, the micelles based on coiled-coil-PEG side conjugates have great potential as nanomedicines, as they are in the appropriate size range and display tremendous stability and robustness over a range of conditions, as demonstrated in Chapter 5.

Various approaches have been developed to generate particles with precise control over particle size, shape, surface characteristics, and disassembly, including dendrimers, shell cross-linked knedel-like particles (SCKs), liposomes, modified quantum dots, and virus-like particles. To date, limited nanoparticles combining just a fraction of the requirements mentioned above have been achieved. Dendrimers mimic
the size, solubility, and shape of human proteins and are viewed as an ideal choice for many therapeutic and diagnostic applications. Dendrimers such as VivaGel® and SuperFect® are under commercial development; however, concerns regarding systemic toxicity impact the choice of building blocks, size distribution, surface termination and the number of conjugated targeting ligands and therapeutic moieties. Synthetic virus-like nanoparticles (SVLPs), generated by virus capsid gene expression, have regular structures with uniform particle sizes and well-controlled ligand presentation. However, significant concerns remain on their safety, potential viral infection, and other possible side effects. From a biomaterials perspective, long capsid proteins cannot be produced by chemical synthesis. Using gene expression, extensive purification is required to avoid immunogenesis. Additionally, the capsid proteins are subject to proteolysis and are difficult to transport and store for long periods of time without refrigeration. Current FDA approved Doxil (~100 nm) and Abraxane (~130 nm), although highly promising with enhanced pharmacokinetics, have provided only modest survival benefits, presumably due to drug leakage during circulation and inefficient diffusion of the particles through tumor tissue. This is attributed to inefficient transport of the chemotherapeutic drug into the tumor due to their relatively larger size and drug leakage during blood circulation.

Amphiphilic molecules spontaneously self-assemble into micelles and are versatile in producing multi-valent, multi-functional and modular small organic particles. Further, amphiphilic components cross cell membranes without a requirement for a specific uptake pathway. Yet, it is challenging to generate useful, stable micelles (that is, micelles with long half-lives for break-up) less than 30 nm in diameter to meet current demands in nanomedicine. Micelles in this size range are generally fluid, dynamic assemblies, where the subunit amphiphiles are constantly exchanging with the surrounding media and with other micelles. The presence of chemical traps that stabilize individual amphiphiles further reduces the stability of the micelles and leads to undesirable cargo leakage and disassembly in vitro. Chemically crosslinking the headgroups and/or engineering multiple pairs of intermolecular interactions among the headgroups can be effective to obtain stable micelles. However, the enhanced stability has led to undesirable accumulation in the liver and spleen, rather than urinary clearance of smaller subunits.

Chapter 5 describes the design, engineering, and physical characterization of highly stable 3-helix micelles, ~15 nm in diameter, with great potential as nanocarriers. In this chapter, the biological evaluation for such applications is described, with studies at both the in vitro cell level, done in house, and in vivo animal models that are done with collaborators at UC Davis (K.W. Ferrara).
§ 7.4 Results and discussion

Biological studies of the micelles were performed in order to determine their potential as drug delivery vehicles. Their behavior at the cellular level was investigated with the following questions in mind:

1) Are the micelles cytotoxic?
2) Do the micelles get uptaken by cells?
3) Can the micelles deliver cargo to cells?
4) Where do the micelles traffic intracellularly?
5) Do the micelles remain assembled inside cells?

*In vitro* cell studies were conducted with NIH/3T3 mouse fibroblasts as a basic healthy cell line for preliminary studies. Figure 7.2 shows cell viability of fibroblasts, measured by the MTT assay, that have been incubated with 20 μM micelle solutions for various durations. Cells show nearly 100% viability for all timepoints measured, indicating that the micelles themselves are noncytotoxic.

![Graph](image)

**Figure 7.2.** Cell viability of NIH/3T3 fibroblasts, assessed by the MTT assay, that have been incubated with 20 μM micelle solutions for various durations.
In order to facilitate further biological evaluations of the 3-helix bundle based micelles, a fluorescent dye, fluorescein, was conjugated to the C-terminus of the peptide for imaging and detection. The synthetic scheme of dye-labeled micelles, which differs slightly from that of unlabeled micelles, is shown in Figure 7.2. Peptides are synthesized and alkyl tails are conjugated on resin using standard Fmoc chemistry, as is typically done of unmodified C-terminal peptides. However, the site-specific conjugation of PEG to the middle of the peptide sequence also occurs on solid phase through selective removal of an Alloc-protecting group on the Lys14 sidechain. Once cleaved off the resin, the thiol of the cysteine at the C-terminus of the peptide is free for conjugation of maleimide end-functionalized dyes in solution post micellar assembly. The labeling efficiency was estimated to be approximately one dye molecule per 3-helix bundle. The low labeling efficiency may possibly result from steric effects.

Figure 7.3. Synthetic scheme of fluorescent dye-labeled amphiphiles. Water-soluble dyes, such as Oregon Green and fluorescein, were conjugated to the C-terminus of peptides.

Figure 7.4 shows the SAXS profiles comparing the form factors of dye-labeled micelles and unmodified micelles. Both curves are very similar, indicating that dye-labeling does not affect micelle assembly. The form factor of the dye-labeled micelles shows a slightly higher degree of polydispersity, indicating possibly a less well-defined shell-solvent interface due to the partial labeling of the peptides. Fitting the profile to a
spherical core-shell form factor yielded a core radius of \(\sim 2.7\) nm and a shell thickness \(\sim 4.2\) nm, which are very similar to the dimensions of the unlabeled micelle.

![Figure 7.4](image)

**Figure 7.4.** SAXS profiles of fluorescein-labeled and unlabeled micelles, demonstrating that conjugation of fluorescent dyes to the surface of the micelle does not change micellar size and shape.

Doxorubicin (Dox), a common hydrophobic anti-cancer drug, was also encapsulated within micelles as a fluorescent marker and as a model drug to determine the effectiveness of the micelle as a drug delivery vehicle. Doxorubicin, which is used as a model therapeutic for our studies, is an anthracycline and works by intercalating DNA. Dox demonstrated facile encapsulation within the micelle at a ratio of \(\sim 1\) Dox molecule per 3-helix bundle via a passive diffusion mechananism post micellar assembly. SEC confirmed the coelution of the micelle and DOX, indicating DOX encapsulation in the micelle. Structural studies showed that the micelle slightly decreased in size upon cargo encapsulation. SEC of Dox-loaded micelles after 1 month of storage showed that they remained stable over this time period, showing no signs of aggregation or DOX leakage. Higher DOX loadings, up to 8 wt\%, can be achieved by co-assembling the drug and the micelle.

Cellular uptake and intracellular trafficking were next investigated. Figure 7.5a shows confocal microscopy images of NIH/3T3 fibroblasts that have been incubated with fluorescent dye-labeled micelles. Nuclei were stained blue with Hoechst dye, lysosomes red with Lysotracker Red, and micelles were green with fluorescein labeling. Overlay of
the red and green channels shows that the micelles are localized to lysosomes and are endocytosed by cells via the endolysosomal pathway within 5 minutes of incubation. Cellular uptake likely occurs through non-specific pinocytosis, as there are no active targeting ligands on the surface of the micelles for these studies. Confocal microscopy shows that micelles remain localized in lysosomes after two hours.

Figure 7.5. Confocal microscopy images of NIH/3T3 fibroblasts that have been incubated with (a) fluorescein-labeled micelles for 5 min; (b) Dox-loaded micelles for 5 min; (c) Dox-loaded micelles for various times; (d) free Doxorubicin. Nuclei are stained blue with Hoechst dye. In the case of micelles labeled with fluorescein, lysosomes are stained red with Lysotracker Red. In the case of Dox-loaded micelles, lysosomes are stained green with Lysotracker Green.
Similarly, cellular uptake was conducted with Dox-loaded micelles, which fluoresce red, and lysosomes were stained green with Lysotracker Green. Overlay of the red and green channels in Figure 7.5b shows that the Dox that was delivered by the micelles were also localized to lysosomes at the 5 minute time point. A time course study shows that it takes 30-45 min for Dox that was delivered by the micelles to reach the nucleus, as shown in Figure 7.5c. In contrast, free Dox localizes to the nucleus immediately, within 5 min, as seen by the overlap of the red and blue channels in Figure 6.5d. This demonstrates that the micelles can serve as a delivery vehicle to tailor the cellular uptake, time release, and intracellular trafficking of drugs. These preliminary studies have demonstrated the baseline for cellular uptake behavior of non-targeted micelles and can serve as a basis of comparison for targeted micelles, which will be investigated in the future. Though unfunctionalized micelles get passively uptaken by all cell lines studied, it is expected that the degree of specific uptake upon conjugation of a targeting ligand would overwhelm the signal seen from the non-specific uptake studied up until this point.

Next, the mechanism by which Dox gets released from micelles and reaches the nucleus was investigated. It has been shown that micelles get endocytosed and are localized to the endolysosomal pathway immediately and remain localized in lysosomes 2 hr after incubation. In order for Dox that is encapsulated in micelles to reach the nucleus, one of two options is possible: either the micelle disassembles, thereby releasing Dox, or the micelle remains assembled and Dox diffuses out of the micelle. Dynamic light scattering (Figure 7.6a) shows that the micelles stay assembled at pH 5, which is the low pH of lysosomes. Dox diffusion out of the micelles as a function of pH was also investigated. Comparison of the release profile at pH 7.4 and pH 5 shows that release is enhanced at low pH, as shown in Figure 7.6b. The same phenomenon has been observed for micelles composed of PEGylated polypeptides, where release of Dox was accelerated by decreasing the pH from 7.4 to 5.58 This was attributed to protonation of the 3’ amine of Dox. Our release profiles follow the same biphasic trend as typical micelles, except the time-scale of release for our system is an order of magnitude slower than other micellar systems. The long times required for drug diffusion are likely related to the high stability of the micelles and the slow kinetics of subunit exchange. The slow timescale for release is not consistent with the in vitro cell experiments, as the diffusion profile shows a significant amount of drug still retained in the micelle at the 30 min time-point at pH 5. Hours are required before a substantial fraction of drugs diffuse out of the micelle. The low pH environment of lysosomes does not appear to be the contributing factor to drug release in vitro.
Because micelles do not disassemble at low pH and there is no substantial drug leakage out of the micelle at low pH on the time scales seen in the cellular uptake experiments, another factor of the lysosomal environment that must be considered is the presence of lysosomal enzymes that are capable of degrading the micelles. For many protein and peptide-based therapeutics, rapid proteolytic degradation by enzymes represents a principal challenge. As discussed in Chapter 3, PEGylation is a common technique for providing protection from proteases because the polymer chains provide a steric barrier and inhibit access to the active site. Proteolysis of the 3-helix micelles was investigated, as it is possible that steric effects provided by the conjugated PEG and the confinement of the micelle structure may inhibit proteolysis of the peptide moieties. Studies have been done to investigate proteolysis of different forms of TAT cell-penetrating peptides using trypsin. Proteolysis kinetics of free peptide TATp, a TATp-conjugate that forms micelles with the peptide exposed on the surface, and a TATp-micelle with PEG providing an exterior shell were compared. Trypsinolysis followed pseudo-first-order kinetics. The cleavage of the free TATp was relatively fast with a half-life of a few minutes ($t_{1/2} \sim 3.5$ min). The TATp conjugate showed more stability with about a 3-fold increase in half-life ($t_{1/2} \sim 10$ min). The peptide in the PEGylated micelle was highly protected against proteolysis with an over 100-fold increase in half-life ($t_{1/2} \sim 430$ min). This shows that micelle assembly does not significantly inhibit proteolysis, but the shielding of PEG on the surface does.

To investigate possible proteolysis of the amphiphiles and subsequent micellar disassembly, the self-quenching of conjugated fluorophores on the surface of the micelle was used as a probe. The fluorescence intensity of fluorescein conjugated to micelles is much lower than the same concentration of fluorescein that is free in solution because the molecules are in such close proximity, as shown in Figure 7.7a. Upon micellar disassembly, fluorescence intensity increases. Fluorescein was chosen because it undergoes self-quenching when conjugated to the surface of micelles at the ratio achieved.
by conjugation post micelle assembly. Oregon Green-labeled micelles synthesized in the same way showed minimal fluorescence self-quenching behavior. Though the pH dependence of fluorescein complicates data analysis, it could not be avoided, as pH-independent dyes are hydrophobic and are not easily conjugated to the surface of micelles. The need for a water-soluble, self-quenching dye made fluorescein the best option for fluorescence recovery experiments.

**Figure 7.** (a) Fluorescence self-quenching of fluorescein when conjugated to the surface of micelles. The concentration of the dye is the same in for both samples. (b) Change in fluorescence intensity of self-quenched fluorescein-labeled micelles over time after incubation with various concentrations of proteinase K, demonstrating the proteolysis of micelles. The rate of degradation increases with the concentration of the protease.

To demonstrate that micelles are subject to proteolysis, fluorescein-labeled micelles were incubated with a broad spectrum protease, proteinase K. Proteinase K cleaves peptide bonds adjacent to the carboxyl group of aliphatic and aromatic amino acids, providing 10 possible cleavage sites in total for 1CW, as shown in Figure 7.8. The fluorescence intensity of the micelle increases quickly several fold upon addition of the protease, as seen in Figure 7.7b. The rate of degradation/disassembly increases as the concentration of the protease increases. MALDI of the micellar solution after addition of the protease confirmed peptide degradation, as the amphiphile is no longer intact. Only PEG is observed because the peptide fragments are smaller than the detection limit. Figure 7.8 shows the mass spectrum of the PEG fragment, and the labeled peak corresponds to the molecular weight of 41 ethylene glycol repeat units conjugated to a small peptide fragment. Studies were then extended to human specific and lysosomal specific proteases, as shown in Figure 7.9. Matrix metalloproteinase is a zinc-dependent endopeptidase that is capable of degrading many extracellular matrix proteins. Cathepsin L is a lysosomal cysteine proteinase and one of the most active endopeptidases found in
the lysosome. It becomes activated at the low pH found in lysosomes, and thus the activity lies almost entirely within those organelles. Fluorescence increase of self-quenched dye-labeled micelles confirmed that both human-specific and lysosomal-specific proteases are also capable of degrading the micelle.

**Figure 7.8.** MALDI-TOF spectra of the micelle solution before and after incubation with proteinase K, verifying the degradation of the peptide and only presence of the PEG after addition of the protease (left). The labeled peak is equal to the mass of 41 ethylene glycol repeat units and a short peptide fragment.

**Figure 7.9.** Change in fluorescence intensity of self-quenched fluorescein-labeled micelles over time after incubation with (a) matrix metalloproteinase-3, a human-specific protease, and (b) cathepsin L, a lysosomal-specific protease.
Figure 7.10. Fluorescein-labeled micelles were incubated with NIH/3T3 fibroblasts for 15 min, and fluorescence intensity was monitored at t = 0, 30, 90 and 180 min. To decouple the pH effects of fluorescein, the same experiment was conducted with fixed cells. These results indicate that micelles are degraded intracellularly.

In order to determine the intracellular fate of the micelles, the change in fluorescence intensity of the micelles was monitored when inside cells. Flow cytometry, which can quantify the fluorescence intensity of individual cells, was used to monitor micelle stability upon endocytosis. Fluorescein-labeled micelles were incubated with NIH/3T3 fibroblasts for 15 min, and fluorescence intensity was monitored at various time-points over the course of the following 3 hrs. The fluorescence intensity of the micelle increased steadily over this time, with a 3-fold increase in intensity at 3 hrs compared to the initial intensity after sample incubation. This is attributed to the un-quenching of dye molecules upon disassembly, most likely due to degradation by lysosomal proteases. To rule out pH effects of fluorescein, the same experiment was conducted with cells that were fixed immediately prior to cytometry at the end of the experiment. Fixation eliminates any differences in intracellular pH, so that any change in fluorescence is due to disassembly and not differences in fluorescence characteristics of
fluorescein as a function of pH. Fixed cells show the same trend of increasing fluorescence intensity over time as live cells, as shown in Figure 6.10, indicating that the micelles do get degraded intracellularly.

Lastly, pharmacokinetic evaluation and biodistribution of the 3-helix micelles were carried out to validate their potential as nanocarriers upon systemic injection. This work was done in collaboration with Dr. K.W. Ferrara at UC Davis. For these studies, 1CW-dC18-PEG2K amphiphiles were used to maximize the stability of the micelle, as demonstrated in Chapter 5. Furthermore, the surface of the micelle was PEGylated with PEG of molecular weight 750 Da to mask the charge and surface of the micelle. The preparation of $^{64}$Cu labeled 3-helix micelles was achieved by co-assembly of metal-chelator functionalized amphiphilic peptides with the regular amphiphilics followed by high affinity coordination reaction with $^{64}$Cu ions. Micelle solutions were administered through intravenous injection to mice bearing NDL tumors. Using positron emission tomography (PET), the pharmacokinetics of radiolabeled micelles were assessed and compared with long circulating liposomes and conventional DSPE-PEG2K micelles. All tested micelles have similar degrees of hydrophobicity, as they are composed of double C18 tails and a stealth layer of PEG to prevent non-specific protein adsorption. PET images were acquired over 48 hours after injection and demonstrated that the 3-helix micelles remained highly concentrated in the blood pool, with minimal accumulation in the liver and spleen (Figure 7.11a). Approximately 15 ± 1.5% ID/g remained circulating in the blood pool even at 48 hours post injection (Figure 7.11b). Based on the image data set, the pharmacokinetics of 3-helix micelles was fitted using a bi-phasic model. The $\beta$-phase blood circulation half-life ($t_{1/2,\beta}$) of the micelles was estimated to be ~29.5 hours (Figure 7.11b), which is comparable to that of successful dendrimers. The activity was confined to plasma rather than the circulating cellular components (Figure 7.11c).

Figure 7.12 shows the comparison of the biodistribution profile of the 3-helix micelles with long circulating liposomes and conventional DSPE-PEG2K-OMe micelles in non-perfused mice. The radioactivity resulting from injection of 3-helix micelles is the highest in the blood pool with 15.0 ± 1.5% ID/g. The uptake of the 3-helix micelles in NDL model tumors (5.7 ± 0.9% ID/g) was similar to that achieved with $^{64}$Cu-liposomes (4.3% ID/g) and $^{64}$Cu-albumin in a similar model (MIN-O) and may be attributed to the EPR effect. The radioactivities of different organs were observed as following: 4.6 ± 0.5% ID/g in the spleen, 4.5 ± 0.2% ID/g in the liver, 2.9 ± 0.3% ID/g in the kidney, and 2.1 ± 0.2% ID/g in the heart. The animals were not perfused in the study. Considering the high activity remained in blood at the point of the biodistribution study, the residual blood in the liver and spleen may partially account for the activities observed in these organs. To further clarify the systemic clearance pathway, radioactivities in the duodenum and jejunum were measured, which are ~ 2% ID/g (Figure 7.12a). The low activity in the digestive system, liver and spleen indicate that the reticulo-endothelial systems (RES) clearance may not be the primary clearance pathway for the 3-helix micelles.
Figure 7.11. In vivo assessment of micelle circulation and stability (a) Coronal (top) and transverse (bottom) view of sliced PET images of $^{64}$Cu-1CW-dC18-PEG2k micelles administered mouse. Images were acquired after the reconstruction of histogram with maximum a posteriori probability (MAP) estimate. (b) Blood radioactivity (%ID/cc) of $^{64}$Cu-1CW-dC18-PEG2k micelles in blood. Curve was fitted as two phase exponential decay ($Y=45.32e^{-0.0235t} + 16.42e^{-1.27t}$, $t_{1/2} \alpha = 0.55$, $\beta = 29.52$). (c) % Radioactivity in plasma and blood cells (48 hours after injection). % Radioactivity was calculated by the following equation [100 x plasma radioactivity/(plasma radioactivity + blood cells radioactivity)].

The radioactivity detected within the blood, liver and spleen was also compared among the 3-helix micelles, DSPE-PEG2K-OMe micelles and long circulating liposomes (Figure 7.12b). Due to the rapid clearance of DSPE-PEG2K-OMe micelles, biodistribution results at 24 hours were used for comparison to those obtained at 48 hours with long circulating liposomes and 3-helix micelles. The radioactivity in the liver resulting from DSPE-PEG2K-OMe micelles remained at a similar level to that of long circulating liposomes. Substantial differences between 3-helix micelles and long circulating liposomes were apparent: blood circulation was extended and liver and spleen accumulation was decreased compared with other two strategies.
Figure 7.12. Biodistribution analysis (%ID/g) of conventional micelles (n=2), $^{64}\text{Cu}$-1CW-dC18-PEG2k micelles (n=6), and long circulating liposomes (n=4) (a) Comparison of radioactivity (%ID/g) of, long circulating liposomes (liposomal 48 h data was obtained from a previous study$^{65}$) and $^{64}\text{Cu}$-1CW-dC18-PEG2k micelles (48 h). (b) Comparison of radioactivity (%ID/g) of conventional micelles, long circulating liposomes and $^{64}\text{Cu}$-1CW-dC18-PEG2k micelles. Statistical analysis between groups was performed with one-way ANOVA followed by Tukey’s multiple comparison test (***, $P<0.0001$, **, $P<0.001$, *, $P<0.05$).

In vivo pharmacokinetics and biodistribution studies clearly demonstrate that 3-helix micelles achieve a long circulation half-life and efficient clearance. The long circulation half-life is attributed to the size, physical stability and surface chemistry of the micelle. Reduced accumulation in the liver, spleen and intestine, combined with urinary activity, suggest that the 3-helix micelles were not primarily cleared through the RES pathway. One hypothesis for the systemic clearance of 3-helix micelles is first by monomer desorption, where individual or trimeric amphiphiles exit the micelle during blood circulation. If the hydrophobic C18 tails cannot be shielded by the headgroup, the amphiphiles will be captured by serum proteins and subsequently cleared by the RES system, similar to results of other micelles, including DSPE-PEG2K and block copolymer based micelles. As the hydrophilic headgroup, i.e. 1CW-PEG2K, is over 5 kDa in molecular weight, it is possible that 1CW-PEG2K may wrap the C18 chains to shield non-favorable interactions between C18 and water, similar to the findings in Chapter 4.$^{63}$ The molecular weight of 1CW-dC18-PEG2K amphiphile is only ~6 kDa, well below the critical molecular weight cutoff to pass through the glomerular membranes. As an alternative clearance mechanism to physical desorption of micelles, the 3-helix micelles may be digested via proteolysis upon internalization by cells, as described above. Once the peptide is enzymatically degraded, the micelle will disassemble and the fragments of the amphiphile will be metabolized.
§ 7.5 Conclusion

We have generated well-defined, monodisperse micelles ~15 nm in diameter that show tremendous potential as nanocarriers due to their stability. Studies at the in vitro cellular level showed that they are capable of tailoring the uptake, intracellular trafficking, and release of drugs. In addition, they are most likely degraded in lysosomes upon endocytosis, which serves as both a clearance mechanism and a drug release mechanism. Studies at the in vivo animal model level have demonstrated a prolonged circulation in the blood and minimal accumulation in the RES compared to other drug delivery counterparts. Such studies have helped to elucidate the behavior of these materials in biological settings, which are required if they are to reach their potential as nanocarriers.

§ 7.6 Experimental

7.6.1 Synthesis of Dye-Labeled Amphiphiles. The peptide was synthesized on solid-phase with an Alloc-protected lysine (Lys15) in the middle of the peptide sequence, rather than the usual cysteine. Cysteine was appended to the C-terminus of peptides to facilitate conjugation of maleimide-functionalized fluorescent dyes, such as maleimide-Oregon Green 488 and maleimide-fluorescein, for presentation on the surface of assembled micelles. After addition of the C6 linker and C16 alkyl tails to the N-terminus of the peptide, the Alloc-protecting group was site selectively removed from resin-bound peptide via treatment with Pd(PPh₃)₄ catalyst (0.2 eq) and radical trapping agent PhSiH₃ (24 eq) in 2 ml of DCM for 30 minutes. The polypropylene reaction vessel was placed on a rotator shaker to facilitate complete mixing. After 30 minutes, resin was thoroughly washed with DCM, and the reaction was repeated two more times. Quantitative Alloc removal was observed. The orthogonal deprotection resulted in a free ε-amine at Lys15 while the rest of the amino acids were fully protected. Carboxy-terminated PEG of molecular weight 2000 g/mol polymers were activated with HCTU (4 eq) and DIPEA (8 eq) in 2 ml of DMF for 30 minutes, and the activated polymer mixture was then added to the reaction vial containing peptide-loaded resin. The reaction was performed at room temperature for 96 hours. Then, the reaction mixture was filtered off the resin and the resin was thoroughly washed with DMF to remove unreacted polymer and other reagents. Finally, the peptide-polymer conjugates were cleaved off the resin and deprotected in 5 ml of 95:2.5:2.5 TFA:water:TIS for 3 hours. The cleavage solution was evaporated under a stream of nitrogen prior to precipitation in cold diethyl ether and collection via centrifugation. Amphiphiles were purified by RP-HPLC, as described in Chapter 6. After lyophilization of the purified product, maleimide-functionalized fluorescent dyes were conjugated to the C-terminus of the peptide at a 20 times excess. Free dye was removed via spin dialysis and size exclusion chromatography. Purified, dye-labeled amphiphiles, which showed a conjugation efficiency of 1 dye molecule per 3-helix bundle, were
subsequently used for biological studies. Cysteine at the C-terminus of 1CW-dC18-PEG2K also allows for the conjugation of 6-BAT-maleimide onto the peptides for PET imaging.

### 7.6.2 Preparation of Doxorubicin (Dox)-Loaded Micelles

Doxorubicin (Dox) was encapsulated in micelles by the passive diffusion method. Dox was dissolved in acetone and subsequently added to a micellar solution in 25 mM pH 7.4 phosphate buffer at a large excess. The solution was stirred for several hours uncapped to facilitate evaporation of acetone. Nitrogen was also blown into the solution to remove remaining acetone. Dox was allowed to diffuse into micelles that were already assembled in buffer for minimal effect on the micelle assembly process. Dox precipitates were removed from the micellar solution via centrifugation. Soluble free Dox was removed via extensive spin dialysis. Resulting solutions of doxorubicin encapsulated micelles were used for biological studies. This passive diffusion method resulted in a drug loading of roughly 1 wt%.

### 7.6.3 Dialysis: Cargo Release.

Release of Doxorubicin from micelles was evaluated over time at pH 7.4 and pH 5 by dialysis. Dox-loaded micelles were placed in a dialysis bag of molecular weight cutoff 3000 Da and dialyzed against 25 mM phosphate buffer at physiological pH (pH 7.4) and the low pH of lysosomes (pH 5). The ratio of Dox to peptide was monitored over time via UV-Vis absorbance.

### 7.6.4 Cell Culture

NIH/3T3 fibroblasts were cultured following the aseptic technique. All cabinets and equipment were sprayed with 70% v/v ethanol (in H2O). Nonsterile equipment was sterilized by autoclaving (120 °C). Sample solutions were filtered prior to testing them in vitro. Vials containing 1 mL of suspension of NIH/3T3 cells were stored under liquid nitrogen at -196 °C. Vials were thawed in water bath at 37 °C, and the defrosted suspension was transferred to a sterile tube containing 9 mL of DMEM with 20% FCS. The cell suspension was centrifuged at 1000 rpm at 20 °C for 5 min. The supernatant was removed by aspiration, and the cells were resuspended in 5 mL of Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% FCS. This suspension was transferred to a 25 cm² flask and incubated at 37 °C, 5% CO2 for 24 h. After this, the media was aspirated, and cells were washed three times with sterile PBS solution to remove nonviable cells. 5 mL of fresh medium was added, and cells were incubated and allowed to grow until they reached 80% confluency. For passage, cells were washed with sterile PBS and trypsinized. After several minutes, an equal volume of media was added, and the cells were subsequently pelleted and resuspended as described above. 50 μL of the cell suspension was then mixed with a 50 μL of trypan blue solution (0.2% w/v in PBS). This solution was placed on a hemocytometer to determine the concentration of viable cells. Cells were reseeded in flasks at a density of 4000 cells/cm².

### 7.6.5 Cytotoxicity Assay

Cytotoxicity was determined using the MTT assay. NIH/3T3 cells were seeded into 96-well plates at a density of 8000 cells per well in 200 μL of
medium and incubated for 24 h. After this time, the media was exchanged, and samples of desired concentrations were added for various times, ranging from 1 hr to 2 days. Then, 20 μL of a 5 mg/mL MTT assay stock solution in PBS was added to each well. After the cells were incubated for 4 h, the medium containing the unreacted MTT was removed carefully. The obtained blue formazan crystals were dissolved in 200 μL/well of DMSO, and UV-Vis absorbance at 490 nm was measured using a Sunrise Tecan Plate Reader. The results were expressed as cell viability relative to control cells containing no sample.

7.6.6 Confocal Microscopy. Confocal microscopy was performed using a Zeiss LSM 510 Meta Laser Scanning Microscope. 8 well glass slides were coated with Cell Tak following the product description. Cells were seeded at low density and allowed to adhere and reach the desired confluency. Cells were incubated with sample, typically 20 μM, for various times and stained with 2 μL of 500 μg/mL Hoechst and 2 μL of 100 μM Lysotracker for 10 min. Cells were fixed with 4% paraformaldehyde for 15 min. Slides were mounted with Fluoromount-G and usually imaged immediately.

7.6.7 Micelle Degradation In vitro. Fluorescein-labeled micelles were incubated with varying concentrations of proteinase K, and the change in fluorescence intensity was monitored over time. Matrix metalloproteinase-3 and cathepsin L were also used as human specific and lysosomal specific proteases.

7.6.8 Flow Cytometry. Flow cytometry was used to measure the degradation/disassembly of the micelle when inside cells. The change in fluorescence intensity associated with each cell as a function of time was monitored. NIH/3T3 fibroblasts were incubated with 20 μM solutions of fluorescein-labeled micelles in PBS for 15 min. Samples were subsequently removed from the media and the cells were washed thoroughly with PBS. Fresh media was added and cells were incubated under standard conditions for various times post sample introduction. After various timepoints, cells were trypsinized, resuspended in cold PBS, and kept on ice until measurement by flow cytometry.

Flow cytometry was performed using a Beckman Coulter. A minimum of 10,000 cells were analyzed for each sample. Fluorescein fluorescence was measured using the 488 nm line from a 15 mW argon ion laser and the green fluorescence channel (FL1).

7.6.9 Synthesis of 6-BAT-maleimide. 6-Aminobenzyl TETA (25 mg) was reacted with sulfo-SMCC (25 mg, ProteoChem, Denver) in phosphate buffered saline (PBS 1x, 8 ml) and the pH was maintained at 7 for 2 hours with the addition of 1 M sodium hydroxide solution. The reaction mixture was diluted with 0.1% TFA solution (4 ml). 6-BAT maleimide was isolated with a reverse phase HPLC system (Jupiter Proteo C12, 250 x 10 mm) and elution was monitored at 220 and 254 nm wavelengths. The flow rate was 3
ml/min and a linear gradient was applied as 5 to 60% solvent B over 30 min (solvent A: 0.1% TFA DI water (v/v), solvent B: 0.1% TFA acetonitrile (v/v)).

7.6.10 Radiolabeling of 1CW-dC18-PEG2K micelles with Cu-64. A lyophilized 1CW-dC18-PEG2K and 6-BAT-1CW-dC18-PEG2K powder (98/2, mol%/mol%, 3.7 mg) was dissolved in deionized water and aged overnight at room temperature. $^{64}$CuCl$_2$ (Isotrace, St. Louis, MO), buffered in 0.1 M ammonium citrate (pH 5.5, 100 ml), was added to a solution of micelles and incubated at 30 °C for 1.5 hours. To remove the nonspecific binding of Cu-64, 0.1 M EDTA (10 µl) was added and the mixture was incubated for 10 min at room temperature. Size exclusion chromatography (Sepadex G-75, GE healthcare) demonstrated Cu-64 labeled micelles with more than 95% labeling yield in a 2 ml volume. Cu-64 micelles were concentrated by centrifugation (4000 g) for 30 minutes. The specific activity of the micelles at the end of synthesis was 140 GBq/mol.

7.6.11 Radiolabeling of conventional micelles with Cu-64. DSPE-PEG2K-OMe and 6-BAT lipid $^{64}$ (97/3, mol%/mol%, 2 mg) in chloroform were dried in a glass test tube under gentle nitrogen stream at 50 °C. Dried lipids were lyophilized overnight. Warmed deionized water (0.5 ml) was added to the test tube, which was gently shaken until the solution became clear. $^{64}$CuCl$_2$ (2.51 mCi), buffered in 0.1 M ammonium citrate (pH 5.5, 100 ml), was added to a solution of micelles and incubated at 30 °C for 1 hour, followed by radiolabeling as in (M21). The labeling yield was 95% and the specific activity of the micelles at the end of the synthesis was 124 GBq/mol.

7.6.12 Animal Protocol (NDL tumor mouse model). All animal experiments were conducted under a protocol approved by the University of California, Davis, Animal Care and use Committee (Davis, CA). Four 4 week old female FVB mice weighing 19-22 g (Charles River, Wilmington, MA) were housed in a temperature controlled room in ventilated cages. All animals were maintained on a 12 hour light cycle and were provided standard rodent chow and water ad libitum. To generate NDL tumors by tumor cell injection, the recipient mice were anesthetized by an IP injection of a ketamine (100 mg/kg)/xylazine (10 mg/kg) solution. Once deeply anesthetized, the animal’s underbelly was shaved with clippers and depilatory cream was applied as necessary for complete fur removal. The injection sites were then cleansed with betadine and eye lubricant was applied to the eyes. A 5 mm incision was made adjacent to the #4 mammary nipples on the right and left sides. The #4 inguinal fat pads were then bluntly dissected and exposed. A solution of 1 x 10$^6$ NDL tumor cells suspended in 20 µl PBS was injected directly into the left and right 4th inguinal mammary fat pads of the recipient mice using a 29 gauge needle. The incision sites were then closed with 1 wound clip per side, and a one-time injection of Buprenex was given for pain management at 0.05-0.1 mg/kg subcutaneously before the animal was ambulatory. The wounds were monitored for 7 days until the wound clips were removed. The tumor was allowed to grow for 12 days before reaching a size of approximately 5mm on the first day of the study.
7.6.13 MicroPET imaging and biodistribution analyses. After the injection of $^{64}$Cu-ICW-dC18-PEG2k micelles, female FVB mice (n=4) bearing NDL tumors bilaterally within the mammary fat pads (M23) were imaged with microPET and the biodistribution assessed. In vivo PET scans were obtained for 30 minutes immediately after tail vein injection of $^{64}$Cu-ICW-dC18-PEG2k micelles (316 ± 83 μCi and 86 ± 24 nmol lipid per mouse) in 150 μL PBS and for 30 min at 3, 6, 24, and 48 h after injection. Animals anesthetized with 2% to 3% isoflurane were placed in pairs on the scanner bed and PET acquisitions were obtained using a small-animal PET scanner (Focus120, Siemens Medical Solutions, Inc.). Animals were euthanized by an IP injection overdose of sodium pentobarbital (150-200mg/kg) and perfused with saline. Briefly, once the animals were euthanized, the chest cavity was opened exposing the heart. A 23gauge needle attached to a perfusion apparatus was inserted into the left ventricle while the right atrium was cut. Saline (20 ml) was allowed to circulate through the body until the liver and kidneys blanched in color. Once perfusion was complete, organs were harvested for biodistribution and the radioactivity measured in a γ-counter (Perkin-Elmer Life Sciences). For the biodistribution of Cu-64 labeled conventional micelles, two female Balb/c mice weighing 26-27 g (Charles River, Wilmington, MA) were used. Cu-64 labeled conventional micelles (198 ± 2 μCi and 69 ± 1 nmol lipids per mouse) were administered via the tail vein, the animals were sacrificed at 24 hours after injection due to the rapid clearance of the radioactivity, and the procedures above followed for biodistribution.
Afterword

Peptide-polymer conjugates clearly hold great promise as an interesting class of materials with applications in nanomedicine and beyond. Because peptide-polymer conjugates are hybrid materials that combine the biological and synthetic worlds, research in this area often lies at the interface between many disciplines. The work described in this dissertation spans the range from fundamental materials science and polymer physics, to chemical biology, to scattering, to cell biology. From a materials science point of view, a fundamental understanding of these hybrids is necessary, along with control over their self-assembly into well-defined nanostructures, if these building blocks are to reach their full potential in biological applications. Because many noncovalent interactions of similar energy scales underlie the behavior of peptide-polymer conjugates, a delicate balance of the various energetic contributions must be achieved to reach targeted assemblies. Detailed structural characterization is also necessary to fully deduce the structure of the peptide and the polymer chain conformation. With fundamental structural and behavioral properties understood, the knowledge gained can then be used to rationally design peptide-polymer conjugates with properties as desired. Many parameters can be tailored to achieve a target, including, but not limited to, the peptide sequence and length, the chemical nature of the polymer, the length of the polymer, the solvent (depending on the application), the site of conjugation (or the architecture of the conjugate), etc.

Future work spans the range from fundamental studies, to self-assembly, to biological applications development. Fundamental questions include systematic variation of each component of the amphiphile to determine the effects on their solution phase behavior and their assembly at the air/water interface. To further drug delivery applications, specific ligands could be appended to the surface of micelles to achieve active targeting. Also, other peptide sequences could be implemented to serve a therapeutic role rather than solely a structural one. The incorporation of stimuli-responsive micellar disassembly or drug release may also be important to bypass lysosomal localization of cargo and effective delivery of therapeutics. Lastly, the use of heterotrimer peptides would allow for the multivalent presentation of multiple chemical cues and functionalities on the surface of micelles for eliciting specific cellular responses.
Appendix

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A.1.1. MALDI-TOF mass spectra of 1CW-PEG750. The distance between two neighboring peaks is 44.05 Da, the mass of an ethylene glycol repeat unit. The labeled peak corresponds to the sum of the masses of 19 ethylene glycol repeat units and the mass of the monodisperse peptide segment.
A.1.2. MALDI-TOF mass spectra of 1CW-PEG5K. The labeled peak corresponds to the sum of the masses of 112 ethylene glycol repeat units and the mass of the monodisperse peptide segment.
A.1.3. MALDI-TOF mass spectra of H10H24-PEG2K. The distance between two neighboring peaks is 44.05 Da, the mass of an ethylene glycol repeat unit. The labeled peak corresponds to the sum of the masses of 44 ethylene glycol repeat units and the mass of the monodisperse peptide segment.
A.1.4. MALDI-TOF mass spectra of H10H24-PEG5K. The labeled peak corresponds to the sum of the masses of 112 ethylene glycol units and the mass of the monodisperse peptide segment.
A.1.5. A wider range of the MALDI-TOF spectrum of 1CW-PEG2K. The labeled peak at 3749 g/mol does indicate the presence of trace amounts of free peptide. Since MALDI-TOF cannot quantify the exact fraction of free peptide, we resolved this by the use of the standard Ellman’s Test. Conservatively, we estimate that at least 95% of peptides was conjugated to PEG during the reaction.
A.1.6 Heme Titration

The plot of absorbance at 412 nm as a function the ratio of heme to 4-helix bundles (Fig. 8 inset) was fit using the following equation:

\[
\text{Abs}_{412\text{nm}} = \text{bg} + \varepsilon_f xB + \frac{1}{2} (\varepsilon_b - \varepsilon_f) \left[ (K_d + xB + B) \pm \sqrt{(K_d + xB + B)^2 - 4xB^2} \right]
\]

where \(\text{bg}\) = background, \(\varepsilon_f\) = extinction coefficient of free heme in solution, \(\varepsilon_b\) = extinction coefficient of bound heme, \(x\) = heme to 4-helix bundle ratio, \(B\) = concentration of 4-helix bundles in solution, and \(K_d\) = dissociation constant. \(B\) was fixed at 1 \(\mu\)M and \(\varepsilon_f\) at 35 000 \(M^{-1}cm^{-1}\). The other variables were allowed to float, yielding \(K_d = 3.06 \mu\)M ± 0.34 \(\mu\)M and \(\varepsilon_b = 4.8e5 \pm 2.46e4 \ M^{-1}cm^{-1}\) for H10H24. For H10H24-PEG2K, \(K_d = 3.44 \mu\)M ± 0.44 \(\mu\)M and \(\varepsilon_b = 4.25e5 \pm 2.63e4 \ M^{-1}cm^{-1}\). For a single bound heme, the \(\varepsilon_b\) is approximately 1.2e5 \(M^{-1}cm^{-1}\). The fitted \(\varepsilon_b\) values indicate that the fitted dissociation constants reflect the incorporation of the third or even fourth heme into the peptide bundles.
A.2 Supporting information for Chapter 3

A.2.1 Guinier analysis of 1CW and 1CW-PEG side-conjugates:

<table>
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<th>1CW</th>
<th>PEG MW</th>
<th>V/mol (cm$^3$/g)</th>
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<tr>
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</tr>
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<td>2000</td>
<td>1.61E+04</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>2.01E+04</td>
</tr>
</tbody>
</table>
A.2.2 Guinier analysis of H10H24 and H10H24-PEG side-conjugates:

<table>
<thead>
<tr>
<th>H10H24</th>
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<th>V/mol (cm^3/g)</th>
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</thead>
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</tr>
<tr>
<td>5000</td>
<td>2.97E+04</td>
<td></td>
</tr>
</tbody>
</table>
A.3.1 Concentration dependence of the percent helicity of 1CW and 1CW-PS1K in pH8 buffer with varying wt% of OG (surfactant). The solid markers represent 1CW solubilized in the following wt% of OG buffers: (●) 0.45% OG, (▲) 0.9% OG, (▼) 1.2% OG. The corresponding unfilled markers represent 1CW-PS1K at each respective OG concentration buffer.
A.3.2. DLS of the OG concentration dependence of 1CW-PS1K (100µM) in pH 8 buffer. The concentration range of OG studied varied from 0 – 2.25 wt%.
A.3.3. Concentration dependence of the percent helicity of H10H24 and H10H24-PS1K in pH8 buffer with varying wt% of OG (surfactant). The solid markers represent H10H24-PS1K solubilized in the following wt% of OG buffers: (▲) 0.45% OG, (■) 0.9% OG, (●) 2.25% OG.
A.3.4. Concentration dependence of the percent helicity of H10H24 and H10H24-PS1K in pH 3 and 5 buffers, with 0.45% OG.
A.3.5. The absorbance at 412 nm (absorbance of heme bound in bundle) vs. the [heme]/[4-helix bundle] ratio determined via heme titration into a 30 µM H10H24-PS1K solution in 0.45wt% OG, pH 8 buffer recorded in a 1 cm path length cuvette by UV-Vis spectrometry. The ratio at which the slope changes in each data set indicates the number of hemes that each bundle binds.
A.3.6. Design of linker to decouple hydrophobicity of PS from steric hindrance

The sequence of H10H24 was designed to have a loop of “GGG” at the N-terminus to relieve any potential steric hindrance. Although H10H24 forms an antiparallel topology based on its crystal structure, Langmuir monolayers of its alkylated analogs showed a parallel topology at the air-water interface. DLS indicates that H10H24 may form micelles in 0.45 wt% OG buffer, so steric hindrance may be enhanced upon micelle formation. To further disseminate the effect of hydrophobicity versus that of steric hindrance, an extended loop was inserted between the peptide helix and the PS to relieve any possible steric hindrance that may have existed. The sequence of the modified peptide, henceforth referred as H10H24-linker, is (Ac-GGGGGKKFEELWLHEELKFEELLKLHEERLKM-CO), with the proposed 6 extra amino acid linker highlighted in bold. The rest of the sequence is otherwise unchanged. CD studies of this new peptide, H10H24-linker, shows helicity of 80% when normalized by the original peptide length, the same helical content as the original peptide, implying that the random coil segment was lengthened without further unwinding of the helix. Upon PS conjugation to this longer sequence, H10H24-linker-PS1K showed similar helical content to that of H10H24-PS1K, with a measured range from ~40-60% helical, as shown in Figure S6. Over this entire helicity range observed for H10H24-linker-PS1K, it was still expected that each bundle would bind two hemes out of the possible four, and this was confirmed experimentally. The titration curves are shown in Figure S7. Because the linker failed to recover the impaired structure and function, the supposition that enthalpy between the peptide and the hydrophobic polymer is the cause, rather than steric hindrance between polymer chains, is clearly strengthened. Combining the results from H10H24-PEG2K, H10H24-PS1K, and H10H24-linker-PS1K, we conclude that for amphiphilic peptide-polymer conjugates, the intermolecular interactions between the peptide and the hydrophobic conjugated polymer is the main cause in the loss of peptide structure, and thus functionality.
A.3.7. CD spectra of ~50μM solutions of H10H24, H10H24-linker, H10H24-PS1K, and H10H24-linker-PS1K in 25mM KH₂PO₄ buffer at pH8, with 0.45% OG. All traces show typical alpha helix characteristics. Inset: Concentration dependence of the percent helicity of (●) H10H24-linker and (▲) H10H24-linker-PS1K in 25mM KH₂PO₄ buffer at pH8, with 0.45% OG.
A.3.8. The absorbance at 412 nm (absorbance of heme bound in bundle) vs. the [heme]/[4-helix bundle] ratio determined via heme titration into a 4 uM peptide solution recorded in a 1 cm path length cuvette by UV-Vis spectrometry for (●) H10H24-linker, (▲) H10H24-linker-PS1K. The ratio at which the slope changes in each data set indicates the number of hemes that each bundle binds.
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Chapter 4


**Chapter 5**


Chapter 6


Chapter 7


