Factors that Influence the Self-Assembly of Amphipathic Peptides

by

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Dedication

In memory of my loving parents, Erwin and Deborah Lee, ehsgo:ge' ae'. 
Biographical Sketch

The author was born in Springville, New York, and was raised on the Seneca Cattaraugus Indian Reservation. She attended Rochester Institute of Technology and graduated with a Bachelor of Science degree in 2005. She began graduate studies in chemistry the summer of 2005 at the University of Rochester. She pursued her research in self-assembling amphipathic peptides under the direction of Professor Bradley L. Nilsson and received the Master of Science degree from the University of Rochester in 2007. She received the Graduate Assistance in Areas of National Need (GAANN) Fellowship that was awarded by the University of Rochester from 2006-2008. She is a member of the American Indian Science and Engineering Society (AISES) and was awarded numerous scholarships through AISES and its affiliates. She joined the New York Army National Guard (NYARNG) in 2009 and serves as a first lieutenant (1LT) in command of a company that supports the war on terrorism in Kuwait and Afghanistan.

The following publication was a result of her work conducted during her doctoral study:

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Lastly, I would like to acknowledge my fellow brothers and sisters in arms that are currently serving in Kuwait and Afghanistan along with all the Soldiers in the 27th Brigade Special Troops Battalion (BSTB).
Abstract

Peptide self-assembly into β-sheet fibrils is of critical importance in amyloid pathology and has been exploited in the design of functional biomaterials. The Ac-(FKFE)$_2$-NH$_2$ peptide is an amphipathic sequence that has been extensively studied and forms putative β-sheet bilayer fibrils. Herein we report explorations of peptide length, sequence order, and aromatic amino acid surface area on self-assembly of Ac-(FKFE)$_2$-NH$_2$-derived peptides. We found that these minute changes have unanticipated and dramatic effects on self-assembly that can be used to tune the self-properties of the resulting assembled materials.

In Chapter 2, we report studies of Ac-(FKFE)$_2$-NH$_2$-derived peptides in which either the N- or C-terminal residue is eliminated. It was found that these simple changes had profound effects on the self-assembly of these peptides. Truncation of the N-terminal residue provided a peptide that assembled into unique materials related to the parent peptide. Truncation of the C-terminal residue gave a peptide that failed to self-assemble.

In Chapter 3, self-assembly of the Ac-(FKFE)$_2$-NH$_2$ peptide was compared to that of four related sequences with varied amino acid sequence patterning. It was found that peptides with alternating hydrophobic/hydrophilic amino acid sequence patterns have a higher propensity to self-assemble relative to peptides in which the hydrophilic and hydrophobic residues are clustered. In addition, sequence patterns had a dramatic impact on the morphology of the assembled materials.

In Chapter 4, the influences of exposed surface area of hydrophobic X residues for Ac-(XKXE)$_2$-NH$_2$ peptides was explored. It was found that increased surface area
enhanced self-assembly propensity for these peptides and also perturbed the structure of the assembled materials.

These findings provide significant insight into the factors that influence the self-assembly of amphipathic peptides. These variations can thus be exploited as variables in the creation of novel materials composed of self-assembled peptides that are tuned for specific applications.
Contributors and Funding Sources

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All experiments conducted within this thesis were performed by the author, Naomi Lee. However, TEM images for Figure 2.4 and Figure 2.7 were taken by Dr. Charles Bowerman, former graduate student in the Nilsson research group at the University of Rochester, Department of Chemistry. All electron diffraction images were obtained from Karen Bentley at the University of Rochester Medical Center electron microscope research core facility.

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Chapter 1

1 Introduction

1.1 Peptide Self-Assembly

Protein self-assembly is a common feature in many biological processes and materials in nature. Regulated self-assembly can result in formation of materials such as silk, wool, and collagen. These materials are formed from highly ordered structures with unique architectures of \( \beta \)-sheet fibrils or triple helical structures; however, protein self-assembly is often associated with amyloid formation. Amyloid proteins can provide a biological function, like hormone storage in the pituitary gland, but these \( \beta \)-sheet aggregates can
lead to specific disease pathologies. Unregulated assembly into amyloid is a causative factor in Alzheimer’s disease, Parkinson’s disease, type II diabetes, and numerous other pathologies.$^{6-11}$ Amyloid fibrils are composed of the cross-$\beta$ architecture in which individual peptides are in an extended $\beta$-strand conformation that orients the amino acid side chains orthogonal to the amide backbone; cross-$\beta$ structures arise when multiple $\beta$-sheets laminate to form fibrils.$^{6,12-14}$ Studying these regulated and unregulated self-assembly processes is important to understanding and manipulating these systems.

Peptide self-assembly has also been exploited in the creation of amyloid-inspired functional biomaterials.$^{15-20}$ Researchers have sought to design minimal length self-assembling peptides that mimic the attractive mechanical features of fibrous or amyloid fibrils, but lack pathological properties. When properly designed, these shorter peptides are capable of forming higher-order structures that are similar to the $\beta$-sheet structures of amyloid fibrils or the triple helical structures of collagen. These bioinspired materials have been applied as scaffolds for tissue engineering,$^{21-30}$ drug delivery vectors,$^{31-39}$ and hydrogels that promote wound healing.$^{40-43}$ These self-assembled materials are attractive since they have the potential to use simple and easily modified peptide sequences that have tunable emergent characteristics. The creation of these materials often relies on empirical or phenomenological approaches. Greater understanding of the mechanisms of peptide self-assembly and the relationship between self-assembly mechanism and the emergent properties of the assembled material are necessary in order to undertake the rational design of self-assembled peptide biomaterials with carefully tuned properties.
1.2 Amphipathic β-Sheet Peptide Self-Assembly

Amphipathic peptides with alternating hydrophobic and hydrophilic residues have an increased propensity to self-assemble into β-sheet fibrils.\textsuperscript{44-46} Although earlier studies of amphipathic self-assembling peptide systems has been reported, Zhang et al. popularized work in this field when demonstrating that amphipathic β-sheet peptides like the Ac-(AEAEAKAK)\textsubscript{2}-NH\textsubscript{2} peptide (EAK16-II) self-assemble into amyloid-like fibrils.\textsuperscript{45-50} Zhang also synthesized altered amphipathic peptides where Glu and Lys were replaced with Arg and Asp residues to create Ac-(RARADADA)\textsubscript{2}-NH\textsubscript{2} peptide sequences that also readily self-assembled into fibrils that form entangled hydrogel networks under physiological conditions.\textsuperscript{45-48,51} These functional hydrogels have been applied as scaffolds for tissue engineering and to promote wound healing.\textsuperscript{28,30,52,53} Schneider et al. have expanded on these types of peptides, designing self-assembling amphipathic β-hairpin peptides of the sequence H\textsubscript{2}N-VKVVKVKV-D\textsuperscript{3}PPT-KVKVKVKVNH\textsubscript{2} (MAX1), utilizing pH to control intramolecular folding of the β-hairpins, which template a turn to enforce intermolecular self-assembly into amyloid-like fibrils.\textsuperscript{22,23,54-57} Aggeli and Boden have developed a series of short linear peptides with the base sequence of Ac-QQRQQQQQQEQQ-NH\textsubscript{2} (P11-1) wherein the Arg and Glu residues were incorporated as complementary Coulombic charges to enforce antiparallel β-sheet formation.\textsuperscript{58-61} Others in the field have also created or manipulated existing model systems for β-sheet peptides in an effort to exploit these systems for biological applications such as drug delivery,\textsuperscript{31-34,62} tissue engineering,\textsuperscript{21-24} and vaccine development.\textsuperscript{63-67} Research has tested the effects of pH,\textsuperscript{68-70} temperature,\textsuperscript{45,71,72} peptide length,\textsuperscript{73-77} ionic strength,\textsuperscript{78,79} hydrophobicity,\textsuperscript{79,80} and concentration\textsuperscript{81-83} on peptide self-assembly. Expanded understanding regarding the
governing principles that regulate self-assembly for these peptides has provided enhanced utility as biomaterials.

The $\beta$-sheet self-assembly of these amphipathic peptides is driven by noncovalent interactions, including hydrophobic, aromatic, ionic interactions, and hydrogen bonding that result in highly-ordered structures. The alternating hydrophobic and hydrophilic pattern of many amphipathic self-assembling $\beta$-sheet peptides easily allows for $\beta$-strand formation of monomer peptide with one face consisting exclusively of the hydrophobic residues and the opposite face containing the hydrophilic residues (Figure 1.1). The $\beta$-strands then self-assemble as a result of noncovalent interactions including hydrogen bonding along the backbone, favorable charge interactions on the hydrophilic face, and hydrophobic/aromatic interactions on the hydrophobic face. The $\beta$-sheets continue to elongate with the backbone orthogonal to the direction of the fibril axis to form cross-$\beta$ fibrils similar to those displayed by amyloid fibrils. The formation of these amyloid-like fibrils with segregated hydrophobic and hydrophilic faces results in the ultimate assembly of a hydrophobic bilayer, in which hydrophobic functionality is buried in the bilayer interior, leaving the hydrophilic side chains solvent-exposed. This bilayer structure produces water-soluble fibrils; thus resulting in a unique feature especially when compared to amyloid fibrils, that are typically insoluble in water. These water soluble fibrils have attractive emergent properties. For example, at high concentrations these soluble fibrils can entangle to form polymer-like hydrogel matrices that have been widely exploited, as described previously.
Figure 1.1. Self-assembly of amphipathic peptides with alternating hydrophobic and hydrophilic amino acids.

Tuning of the emergent properties of these amphipathic β-sheet structures (such as hydrogel rigidity and mechanical strength) are dependent on efficient peptide design and an understanding of how sequence and self-assembly properties are related. Despite abundant literature describing inventive applications of materials derived from self-assembled peptides, significant questions regarding these materials persist. High-resolution structural details of amyloid and amyloid-like materials has been challenging to obtain, but recent advances in solid state NMR are facilitating a revolution on this front. However, no high-resolution structural information that defines peptide packing in amphipathic peptide materials has been reported. Other fundamental questions regarding the relationship between primary peptide sequence, biophysical characteristics, and self-assembly propensity remain. Thus, additional research is needed to address current lack of understanding.

1.3 The Ac-(FKFE)$_2$-NH$_2$ Peptide as a Model to Study Self-Assembly.

The Ac-(FKFE)$_2$-NH$_2$ peptide has been extensively studied as model to understand the self-assembly of amphipathic peptides. Marini et al. found that Ac-(FKFE)$_2$-NH$_2$ rapidly self-assembles to form a dense network of left handed helical fibrils in water at acidic pH. These helical tapes relaxed over time to form flat tapes that were
micrometers in length\textsuperscript{94} and a width of 7-8 nm, that corresponded to the width of two edge-to-edge $\beta$-strands.\textsuperscript{96} Molecular modeling suggests the peptide strands form antiparallel bilayer $\beta$-sheets that are organized with the $N$-terminal amino acid out of register, leaving a dangling $N$-terminal phenylalanine (Phe) exposed to the solvent (Figure 1.2).\textsuperscript{94} While this structure leaves unsatisfied hydrogen bond donors and acceptors as well as exposed hydrophobic functionality, it is required in order to enforce segregated hydrophobic/hydrophilic faces and complementary Coulombic interactions that are required for bilayer formation.

![Proposed structural architecture for amphipathic $\beta$-sheet peptides with the Ac-(XYXZ)$_2$-NH$_2$ motif that results in an out-of-register hydrophobic (X) residue to be unpaired between $\beta$-strands (highlighted by rectangles).](image-url)

**Figure 1.2.** Proposed structural architecture for amphipathic $\beta$-sheet peptides with the Ac-(XYXZ)$_2$-NH$_2$ motif that results in an out-of-register hydrophobic (X) residue to be unpaired between $\beta$-strands (highlighted by rectangles).

Our lab has conducted several studies examining the fundamental mechanisms of Ac-(FKFE)$_2$-NH$_2$ self-assembly. The first of these explored global substitutions along the hydrophobic face, replaced Phe with leucine (Leu), valine (Val), alanine (Ala), and the nonnatural amino acid cyclohexylalanine (Cha).\textsuperscript{80} The purpose of this study was to
determine whether aromatic or hydrophobic interactions were a primary driving force for peptide self-assembly. There had been significant debate regarding the relative contributions of aromatic $\pi-\pi$ interactions vs. more general hydrophobic effects in peptide self-assembly processes, and our substitution analysis enabled an assessment of the effects of increasing hydrophobicity while varying aromatic content. These studies indicated that self-assembly propensity correlates more directly to peptide hydrophobicity regardless of aromatic/nonaromatic character. However, aromatic content does result in self-assembled materials with unique morphologies relative to peptides in which the hydrophobic amino acids are nonaromatic.

Modifications were also made to the hydrophilic face of Ac-(FKFE)$_2$-NH$_2$ by synthesizing variants with all the hydrophilic residues are substituted with lysine (Lys) (Ac-(XKXXK)$_2$-NH$_2$). This substitution introduced molecular frustration that prevented self-assembly of the resulting peptides at low ionic strength due to charge repulsion between positively charged Lys residues. Self-assembly can be promoted by increasing the ionic strength of the solution by addition of NaCl; at high ionic strength, charge-screening enables efficient self-assembly. We prepared a series of Ac-(XKXXK)$_2$-NH$_2$ peptides in which X was replaced with valine (Val), isoleucine (Ile), phenylalanine (Phe), pentafluorophenylalanine (F$_5$-Phe), or cyclohexylalanine (Cha), (in order of increasing hydrophobicity). It was found that the required ionic strength needed to promote self-assembly decreased as peptide hydrophobicity increased. Self-assembly propensity was insensitive to aromatic content in these studies, indicating that self-assembly propensity more directly correlates to hydrophobic, and not aromatic, character of the hydrophobic functionality in these amphipathic peptides.
The β-sheet self-assembly into fibrils can also be influenced by the physical constraints of the peptides. Preparation of Ac-C(FKFE)\textsubscript{2}CG-NH\textsubscript{2} peptides that were cyclized via intramolecular disulfide bond formation between the flanking Cys residues resulted in complete abrogation of β-sheet formation and self-assembly.\textsuperscript{97} Addition of chemical reducing agents results in reduction of the disulfide bond, relaxation to linear β-sheet conformations, and rapid self-assembly into β-sheet fibrils. These studies indicate the necessity of β-sheet structure in self-assembly of amphipathic peptides and provide a method for stimulus responsive self-assembly that is sensitive to biological microenvironments.

We also recently discovered that mixtures of enantiomeric amphipathic peptides efficiently coassemble into hybrid nanofibrils composed of alternating enantiomeric sequences consistent with Pauling’s prediction of “rippled β-sheet” structures.\textsuperscript{98} Specifically, equimolar mixtures of L- and D-(FKFE)\textsubscript{2} peptides selectively coassemble into rippled β-sheet fibrils rather than self-sort into enantiomeric all-L and all-D fibrils.\textsuperscript{99} Isotope-edited infrared and fluorescence FRET spectroscopy support the coassembled rippled β-sheet packing orientation. Isothermal titration calorimetry indicates that formation of coassembled rippled β-sheet fibrils is thermodynamically preferred compared to formation of self-assembled all-L and all-D fibrils. Rippled β-sheet fibrils are a fundamentally new class of engineered peptide biomaterial that increases the complexity that can accessed in the design of noncovalent peptide architectures.

The Ac-(FKFE)\textsubscript{2}-NH\textsubscript{2}-derived amphipathic peptides have also been utilized to create functional amyloid-inspired materials that have the ability to abrogate or enhance the infectivity of HIV-1.\textsuperscript{66,67} Fibrils formed from peptides of the sequence Ac-K\textsubscript{n}(XKXE)\textsubscript{2}-
NH$_2$ (where X = Phe or Cha and n = 2, 4) have been shown to increase HIV-1 infectivity in a similar fashion to naturally occurring amyloid fibrils found in semen called semen-derived enhancer of viral infection (SEVI), which has been shown to drastically increase HIV-1 infectivity by several orders of magnitude. SEVI and Ac-K$_n$(XKXE)$_2$-NH$_2$ fibrils are both cationic β-sheet materials that create a “cationic bridge”, allowing the negatively charged HIV-1 virion to come into proximity of the negatively charged host cell. It was also shown that the fibrillar structure is necessary to induce increased infectivity because cationic peptides that were unable to assemble were not able to increase infectivity. Further, if the N-terminal Lys residues were replaced with Glu residues, and the fibrils were incubated with SEVI, the SEVI-mediated enhancement of infection was completely abrogated. These data imply that carefully constructed amphipathic self-assembling peptides are capable of acting like biologically relevant peptides, and are also able to act as potential microbicides.

These studies have provided significant insight into the fundamental mechanisms of peptide self-assembly and how this relates to the properties of the assembled materials. However, for true bottom-up design of biomaterials, the ability to rationally tune a peptide and predict its self-assembly characteristics is essential. To gain a more complete understanding of the structure/function relationship, further studies of Ac-(FKFE)$_2$-NH$_2$ variants are required. The objective of this thesis is to report the examination of three specific aspects of amphipathic peptide self-assembly as described in the following section. The Ac-(FKFE)$_2$-NH$_2$ peptide is again adopted as a model for the studies reported herein.
1.4 Thesis Objectives

The objective of this work presented in this thesis was to investigate three fundamental factors that influence self-assembly of amphipathic peptides. The first was to determine the impact of minor changes to peptide length on self-assembly propensity by truncation of a single amino acid at either the N- and C-terminus of Ac-(FKFE)\(_2\)-NH\(_2\). Next, we investigated the role of sequence order by synthesizing Ac-(FKFE)\(_2\)-NH\(_2\) and four derivatives with identical amino acid composition but varying primary sequence patterns. Lastly, we probed the volume capacity of the putative hydrophobic bilayer of Ac-(XKXE)\(_2\)-NH\(_2\) self-assembled materials using natural and nonnatural amino acids with increasing hydrophobicity, aromaticity, and molecular volume compared to phenylalanine. Each of these modifications affected peptide self-assembly and provided further insight into fundamental determinants of peptide self-assembly.

1.4.1 Sequence length determinants for self-assembly of amphipathic $\beta$-sheet peptides.

Several published reports indicate that the length of self-assembling amphipathic peptides influences both self-assembly propensity and the properties of the assembled materials. A series of amphipathic peptides developed by Zhang et al. showed that modification of the peptide length has a direct impact on self-assembly propensity. For instance, the Ac-(AEAEAKAK)\(_2\)-NH\(_2\) peptide from the EAK\(_n\) derivatives readily assembles into $\beta$-sheet bilayer fibrils.\(^{28,46-49,51,68}\) However, reducing the number of repeating units abrogate self-assembly for the eight-residue variant, Ac-AEAEAKAK-NH\(_2\) (Table 1.1). The dependence on length was also observed for the RAD\(_n\) peptides in which peptides containing 16 residues self-assembled, but the truncated variants remained unstructured.\(^{28,47-49,51,68}\) The same was not true for peptides composed of the
more hydrophobic phenylalanine residue, where $\beta$-sheet formation was observed for peptides as short as eight-residues.$^{74,94,95,100}$ These data support the hypothesis that both peptide length and amino acid composition influence secondary structure; however, these studies focused on the change in peptide length by varying the number of tetra-amino acid repeats for $\text{Ac-}(\text{FKFE})_n\text{-NH}_2$ (where $n$ is 2, 3, or 4). Therefore, the role of peptide length in self-assembly needs to be assessed using smaller single amino acid changes to the peptide length.

<table>
<thead>
<tr>
<th>Name</th>
<th>Peptide Sequence</th>
<th>Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAK8</td>
<td>$\text{Ac-}\text{AEAEAKAK-NH}_2$</td>
<td>disordered</td>
<td>$^{46,48,49}$</td>
</tr>
<tr>
<td>EAK16</td>
<td>$\text{Ac-}(\text{AEAEAKAK})_2\text{-NH}_2$</td>
<td>$\beta$-Sheet</td>
<td>$^{46,48,49}$</td>
</tr>
<tr>
<td>RAD8</td>
<td>$\text{Ac-RARADADA-NH}_2$</td>
<td>disordered</td>
<td>$^46$</td>
</tr>
<tr>
<td>RAD16</td>
<td>$\text{Ac-}(\text{RARADADA})_2\text{-NH}_2$</td>
<td>$\beta$-Sheet</td>
<td>$^{46,48}$</td>
</tr>
<tr>
<td>KFE8</td>
<td>$\text{Ac-}(\text{FKFE})_2\text{-NH}_2$</td>
<td>$\beta$-Sheet</td>
<td>$^{46,74,75}$</td>
</tr>
<tr>
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<td>$\text{Ac-}(\text{FKFE})_3\text{-NH}_2$</td>
<td>$\beta$-Sheet</td>
<td>$^{46,74,75}$</td>
</tr>
<tr>
<td>KFE16</td>
<td>$\text{Ac-}(\text{FKFE})_4\text{-NH}_2$</td>
<td>$\beta$-Sheet</td>
<td>$^{46,74,75}$</td>
</tr>
<tr>
<td>VK9</td>
<td>$\text{VKVKVKVKJ-NH}_2$</td>
<td>$\beta$-Sheet</td>
<td>$^91$</td>
</tr>
<tr>
<td>VK10</td>
<td>$\text{VKVKVKVKVK-NH}_2$</td>
<td>disordered</td>
<td>$^91$</td>
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<tr>
<td>VK11</td>
<td>$\text{VKVKVKVKVKVJ-NH}_2$</td>
<td>$\beta$-Sheet</td>
<td>$^91$</td>
</tr>
<tr>
<td>VK12</td>
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<td>disordered</td>
<td>$^91$</td>
</tr>
<tr>
<td>VK13</td>
<td>$\text{VKVKVKVKVKVKVJ-NH}_2$</td>
<td>$\beta$-Sheet</td>
<td>$^91$</td>
</tr>
</tbody>
</table>

Schneider et al. have also explored the relationship between peptide length and $\beta$-sheet propensity using an amphipathic peptide consisting of repeating valine and lysine residues. The $(\text{VK})_n\text{-NH}_2$ peptide showed an interesting correlation to peptide length and $\beta$-sheet propensity (Table 1.1).$^{91}$ It was observed that variants with an odd-number of amino acids self-assembled while variants with an even-number of amino acids remained disordered.$^{91}$ Sequence length clearly influences peptide self-assembly in a subtle manner but the fundamental reasons for Schneider’s observations on not clear.
Based on the observation that Ac-(FKFE)$_2$-NH$_2$ packs into fibrils that feature a dangling N-terminal phenylalanine, we proposed that truncation of this sequence by a single amino acid would have minimal effects on the self-assembly propensity of this peptide. The even-numbered Ac-(FKFE)$_2$-NH$_2$ peptide has an antiparallel out-of-register orientation; therefore, truncation by a single amino acid at the N- or C-terminus will result in an odd-numbered peptide (Figure 1.3). This change in peptide length can potentially shift the strand alignment to be in-register for maximum hydrogen bonding donor/acceptor pairs. By maximizing noncovalent interactions, the resulting biomaterials are assumed to be unaffected by this minute change in peptide length. Chapter 2 reports the outcome of these studies.

Figure 1.3. Comparison of even-numbered amphipathic peptides that are out of register vs. odd-numbered peptides that are in-register.
1.4.2 Effects of varied sequence pattern on the self-assembly of amphipathic peptides.

Peptides with identical amino acid composition can have varying self-assembling propensities. The Ac-(RA)$_4$(DA)$_4$-NH$_2$ peptide readily undergoes self-assembly into β-sheet fibril structures while the Ac-(AD)$_4$(AR)$_4$-NH$_2$ transitions from β-sheet to β-helical despite the same amino acid composition.$^{46,47}$ Extremely different morphologies were observed for amphipathic peptides consisting of alternating alanine, glutamic acid, and lysine residues. The Ac-(AE)$_4$(AK)$_4$-NH$_2$ peptide formed globular protein structures while its sequence variant, Ac-(AEAEAKAK)$_2$-NH$_2$, assembled in typical fibrillar structures.$^{(54,60,61)}$ Lastly, the location of the bulkier side chains also affected the fibril morphology. Placing steric bulk within the center of the β-strand resulted in fibrils of a slightly thinner width for GEAEVKIKIEVEAKGK compared to its derivative where the larger side chains of isoleucine (I) and valine (V) were at the N- and C- terminus.$^{101}$ It is evident that sequence order affects the self-assembling propensity along with the morphology of the assembled materials.

We investigated the importance of sequence order for self-assembly and fibril morphology for Ac-(FKFE)$_2$-NH$_2$. The alternating hydrophobic and hydrophilic residues result in a β-sheet bilayer for peptides Ac-(FKFE)$_2$-NH$_2$ and Ac-(FK)$_2$(FE)$_2$-NH$_2$ (Figure 1.4). These peptides are proposed to be out of register because of their primary sequence. Therefore, three derivatives were designed with potential to maximize noncovalent interactions through an in-register strand orientation; however, this modification results in hydrophobic residues being placed on both faces of the β-strand. The first variant, Ac-KEFFFFKE-NH$_2$, concentrates the hydrophobic residues within the center of the strand. Ac-(KFFE)$_2$-NH$_2$ disrupts the hydrophobic core of Ac-KEFFFFKE-NH$_2$ by inserting two
hydrophilic residues between phenylalanine residues. Lastly, the Ac-FFKEKEFF-NH$_2$ has all the hydrophobic residues divided at the N- and C-termini with the hydrophilic residues within the center of the $\beta$-strand. Therefore, these derivatives will investigate the importance of alternating hydrophobic and hydrophilic amino acids often used for $\beta$-sheet formation versus improved cross-strand hydrogen bonding.

**Figure 1.4.** Proposed self-assembly architecture and peptide variants with differing sequence orders for Ac-(FKFE)$_2$-NH$_2$. Self-assembly of amphipathic peptides with alternating hydrophobic and hydrophilic residues into a $\beta$-sheet bilayer are out of register for (A) Ac-(FKFE)$_2$-NH$_2$ and (B) Ac-(FK)$_2$(FE)$_2$-NH$_2$. The suggested strand alignment is in-register for the remaining derivatives (C) Ac-KEFFFFKE-NH$_2$ (D) Ac-(KFFE)$_2$-NH$_2$ (E) and Ac-FFKEKEFF-NH$_2$.

### 1.4.3 Capacity for increased surface area in the hydrophobic core of $\beta$-sheet peptide bilayer nanoribbons.

Studies have been conducted to determine the influence of hydrophobicity and aromaticity on self-assembling Ac-(XKXE)$_2$-NH$_2$ peptides. Previously, it was hypothesized that aromatic side chains are responsible for an increased propensity to self-assemble. It was shown that aromatic residues are not required, but rather a degree of hydrophobicity is needed for self-assembly to occur. The least hydrophobic Ac-(AKAE)$_2$-NH$_2$ fails to form highly ordered structures but replacing the alanine residues
with more hydrophobic Val, Ile, Phe and Cha amino acids rescued self-assembly.\textsuperscript{80} A similar pattern was observed for the Ac-(VK)$_4$-NH$_2$ peptide when more hydrophobic aliphatic and aromatic residues were substituted for Val.\textsuperscript{79} Therefore, it is understood that an increase in hydrophobicity correlates to an increased propensity to self-assemble.

Based on the notion that a minimum hydrophobicity is needed for Ac-(XKXE)$_2$-NH$_2$ peptides, we hypothesized that peptides with an increased surface area and hydrophobicity compared to phenylalanine will also self-assemble, although there may be limits to the steric packing capacity of the hydrophobic core. A series of natural and nonnatural aromatic amino acids were used to assess this hypothesis; phenylalanine (Phe), homophenylalanine (Hph), tryptophan (Trp), 1-napthylalanine (1-Nal), 2-napthylalanine (2-Nal), and biphenylalanine (Bip).\textsuperscript{102} By increasing the side chain surface area, we could also probe the maximum molecular volume that could be tolerated within the hydrophobic bilayer (Figure 1.5).
Figure 1.5. Increasing the side chain surface area for Ac-(XKXE)$_2$-NH$_2$ peptides using natural and nonnatural phenylalanine (Phe), homophenylalanine (Hph), tryptophan (Trp), 1-napthylalanine (1-Nal), 2-napthylalanine (2-Nal), and biphenylalanine (Bip) amino acids.
1.5 Conclusion

In conclusion, this thesis highlights work that investigates three fundamental factors that influence the self-assembly of amphipathic peptides. We focused on minor changes to peptide length by deletion of a single amino acid at the \( N \)- and \( C \)-terminus. Then we assessed the importance of sequence order and registry on amphipathic \( \beta \)-sheet sequences with patterns that are not strict repeats of hydrophobic/hydrophilic pairs. Lastly, we probed the aromatic surface area within the hydrophobic bilayer using a combination of natural and nonnatural amino acids. The results from these studies will be discussed in the following chapters.
1.6 References


Chapter 2

2 Sequence Length Determinants for Self-Assembly of Amphipathic $\beta$-Sheet Peptides

2.1 Abstract

Peptide self-assembly into $\beta$-sheet fibrils is of critical importance in amyloid pathology and has been exploited in the design of functional biomaterials. Amphipathic peptides composed of alternating hydrophobic and hydrophilic amino acids are a privileged class of peptide that have a high propensity to self-assemble into $\beta$-sheet fibrils. The Ac-(FKFE)$_2$-NH$_2$ peptide is an amphipathic sequence that has been
extensively studied and forms putative $\beta$-sheet bilayer fibrils in which the hydrophobic Phe side chains are organized to a single face of each constituent sheet; upon bilayer formation these hydrophobic benzyl groups are sequestered in the hydrophobic core of the resulting fibril. In order for the Phe side chains to be uniformly displayed on one face of Ac-(FKFE)$_2$-NH$_2$ $\beta$-sheets, an antiparallel packing orientation in which one amino acid residue is unpaired (leaving unsatisfied hydrogen bond donor/acceptors and hydrophobic groups) must be adopted. Based on molecular models, we hypothesized that truncated seven-amino acid derivatives of Ac-(FKFE)$_2$-NH$_2$ in which either the $N$-terminal Phe residue (Ac-KFEFKFE-NH$_2$) or the C-terminal Glu residue (Ac-FKFEFKF-NH$_2$) is eliminated should readily self-assemble into $\beta$-sheet bilayers; that allows for all hydrogen bond and hydrophobic/charge interactions to be satisfied. We found, however, that these minute changes in peptide sequence have unanticipated and dramatic effects on the self-assembly of each peptide. Ac-FKFEFKF-NH$_2$ self-assembled into fibrils with unique morphology relative to the parent peptide whereas the Ac-KFEFKFE-NH$_2$ peptide had a strongly reduced propensity to self-assemble, even failing to self-assemble altogether under some conditions. These findings provide significant insight into the effect of sequence length and strand registry as well as hydrophobicity and charge on the self-assembly of simple amphipathic peptides and illuminate the possibility of tuning self-assembly processes and the resulting structures with minute changes to peptide sequence.
2.2 Introduction

Amphipathic peptides with alternating hydrophobic and hydrophilic amino acids can self-assemble into highly ordered amyloid-like fibril structures. These assemblies are organized into putative bilayer nanoribbons in which the hydrophobic side chain groups are sequestered in the nonpolar core of the bilayer (Figure 2.1). The surface of the resulting nanoribbons is thus decorated with hydrophilic functionality, providing fibrils that have a high solubility in water. This property has enabled the use of these fibrils as functional biomaterials that include hydrogel matrices for tissue engineering, immunoadjuvants, therapeutic delivery agents, and tools to study and perturb viral infectivity. Despite a growing number of creative applications for materials derived from self-assembled amphipathic \( \beta \)-sheet peptides, questions remain regarding the relationship between peptide sequence, self-assembly propensity, and the emergent properties of the resulting self-assembled materials.

**Figure 2.1.** Putative architecture of self-assembled nanoribbons derived from the representative Ac-(FKFE)\(_2\)-NH\(_2\) amphipathic peptide. \( \beta \)-Strand monomer peptides self-assemble into a putative \( \beta \)-sheet bilayer in which hydrophobic side chain functionality is buried in the interior of the bilayer.
Sequence length is one parameter that has been shown to affect both self-assembly propensity and the emergent properties of the assembled materials.22-26 For example, amphipathic Ac-(RADA)$_4$-NH$_2$ and Ac-(AKAE)$_4$-NH$_2$ peptides undergo favorable self-assembly into fibrils that entangle to form a hydrogel network.27-30 Conversely, it has been found that similar sequences with shorter repeat lengths such as Ac-(AKAE)$_2$-NH$_2$ have a lower propensity to self-assemble (although replacing Ala with more hydrophobic amino acids can elicit self-assembly at shorter sequence lengths).23,31,32 The length of amphipathic peptide also influences the emergent properties, like hydrogel rigidity, of the resulting self-assembled materials. The Ac-(FKFE)$_2$-NH$_2$ and Ac-(FKFE)$_3$-NH$_2$ peptides have been shown to undergo self-assembly to form fibril hydrogel networks; the hydrogel rigidity declined as the sequence length was reduced.24 Thus, increased sequence length clearly correlates with an enhanced capacity for amphipathic peptide self-assembly and with perturbed corresponding emergent properties for the assembled materials.

Schneider et al. have shown that amphipathic peptide self-assembly is influenced by more subtle changes to length. They found that (VK)$_n$-NH$_2$ peptides (with an even number of amino acids) failed to self-assemble into hydrogel networks of entangled fibrils while (VK)$_n$V-NH$_2$ peptides (where $n = 4, 5, 6$ with an odd number of amino acids) effectively self-assembled into hydrogels.33 Shorter length (VK)$_n$V-NH$_2$ peptides ($n = 4, 5, 6$) also had attenuated self-assembly kinetics and weaker hydrogel rigidity for the resultant self-assembled biomaterials compared to longer variants.

The putative structure of self-assembled materials derived from amphipathic tetrad repeat peptides such as (XKXZ)$_n$ (where $X$ is a hydrophobic residue and $Z$ is a hydrophilic residue) suggests an explanation for why self-assembly of these peptides may
be sensitive to more subtle changes in length. The proposed strand registry for assembled nanoribbons of the Ac-(FKFE)\textsubscript{2}-NH\textsubscript{2} peptide features an antiparallel β-sheet strand registry that is shifted one amino acid out of register.\textsuperscript{23,34} Molecular modeling indicates that the N-terminal Phe residue is most likely the out of register amino acid in these β-sheets.\textsuperscript{28,35,36} The out of registry alignment, despite leaving unsatisfied hydrogen bond donors/acceptors and side chain hydrophobic/charge interactions, is required to form β-sheets that have complementary Coulombic interactions and uniform hydrophobic/hydrophilic faces that ultimately laminate into bilayer nanoribbons. All amphipathic self-assembling peptides of the (XKXZ)\textsubscript{n} motif with an even number of amino acids most likely adopt this out of register packing mode. We predicted that subtle changes to amphipathic peptide length by truncation or addition of a single amino acid to provide a peptide with an odd number of residues should provide sequences that can self-assemble into in-register β-sheet bilayer nanoribbons, satisfying all possible hydrogen bond and hydrophobic/charge interactions with the resulting sheets.

In order to test this hypothesis, we have characterized the self-assembly of Ac-(FKFE)\textsubscript{2}-NH\textsubscript{2} derived peptides that have been shortened by a single amino acid. Specifically, we compared the self-assembly of Ac-(FKFE)\textsubscript{2}-NH\textsubscript{2} to peptides truncated by either the C-terminal Glu (Ac-FKFEFKF-NH\textsubscript{2}) or the N-terminal Phe residue (Ac-KFEFKF-NH\textsubscript{2}). While we predicted that each of these peptides would effectively self-assemble, we instead found that these subtle changes in peptide length had dramatic effects on self-assembly. The parent peptide, Ac-(FKFE)\textsubscript{2}-NH\textsubscript{2}, rapidly assembled into characteristic β-sheet nanoribbons (8.2 ± 1.2 nm nm in diameter) with a left-handed helical twist, as previously reported. The C-terminal Glu-truncated peptide, Ac-
FKFEFKF-NH₂, assembled into distinct assemblies: mixtures of thin fibrils (3.7 ± 0.7 nm in diameter) and much broader 19.2 ± 3.8 nm diameter nanotapes. Conversely, the N-terminal Phe-truncated Ac-KFEFKF-NH₂ peptide failed to self-assemble altogether in unbuffered water. Only when dissolved at neutral pH did the Ac-KFEFKF-NH₂ peptide self-assemble into broad nanotape structures. Simple one amino acid changes in Ac-(FKFE)₂-NH₂-derived peptides thus have profound effects on both self-assembly propensity and on the morphology of the assembled materials. While the structural basis for these observations is not completely understood, these results indicate that the fundamental properties of materials derived from self-assembled amphipathic peptides can be tuned by exceedingly minor changes to the sequence of the assembly motif.

2.3 Results and Discussion

2.3.1 Experimental rationale and peptide design.

As stated in the Introduction, the putative bilayer structure for self-assembled amphipathic Ac-(FKFE)₂-NH₂ fibrils requires adoption of an out-of-register antiparallel β-strand alignment of peptides within the constituent β-sheets. Theoretical studies indicate that the N-terminal Phe residue is most likely out of register for Ac-(FKFE)₂-NH₂ β-sheet fibrils (Figure 2.2A).⁵⁵,⁵⁶ This packing architecture leaves unsatisfied hydrogen bond donors and acceptors as well as unpaired hydrophobic/aromatic groups at the edges of the β-sheet structures. However, this packing mode is favorable because it enables uniform sequestration of hydrophobic and hydrophilic functionality to opposite faces of the resulting β-sheets and also favorably aligns complementary cross-strand charges between Lys and Glu side chains on the exposed hydrophilic face.
Figure 2.2. β-Sheet packing structure of Ac-(FKFE)$_2$-NH$_2$ and related peptides that have been truncated by a single amino acid at either the N- or C-terminus relative to the parent peptide. (A) Ac-(FKFE)$_2$-NH$_2$ with an unpaired dangling Phe residue (shown in red) at the sheet edges. (B) Ac-FKFEFKF-NH$_2$ derived from the parent peptide by truncation of the C-terminal Glu residue shown in an in-register, antiparallel β-sheet packing structure. (C) Ac-KFEFKF-NH$_2$ derived from the parent peptide by truncation of the N-terminal Phe residue shown in an in-register, antiparallel β-sheet packing structure.
Theoretically, amphipathic peptides with an odd number of amino acids should be able to self-assemble into β-sheet fibrils that lack dangling residues at the sheet edges and in which all hydrogen bond and hydrophobic/ aromatic pairings are satisfied. For example, we constructed β-sheet models of amphipathic peptides derived from Ac-(FKFE)$_2$-NH$_2$ that illustrate that subtle changes to the length by deletion of a single amino acid at either the C- or N- terminus gives sequences that should be competent to self-assemble (Figure 2.2B-C). These seven amino acid peptide sequences, Ac-FKFEFKF-NH$_2$ (Figure 2.2B) and Ac-KFEFKFE-NH$_2$ (2.2C), are Ac-(FKFE)$_2$-NH$_2$ derivatives that have been truncated at the C-terminal Glu residue or the N-terminal Phe residue, respectively. The Ac-FKFEFKF-NH$_2$ peptide can form in-register antiparallel β-sheet assemblies, although electrostatic repulsion may exist (dependent on pH) between side chain functionality on the hydrophilic face based on putative cross-strand pairing of like charges (Glu paired with Glu, Lys paired with Lys).$^{37,38}$ The Ac-KFEFKFE-NH$_2$ peptide can also pack into an in-register antiparallel β-sheet; however, the putative packing structure for this peptide maintains complementary cross-strand pairing of negatively charged Glu and positively charged Lys residues. Based on these models, we hypothesized that both truncated peptides, Ac-FKFEFKF-NH$_2$ and Ac-KFEFKFE-NH$_2$, should be competent to self-assemble into β-sheet fibrils similar to those formed by the parent Ac-(FKFE)$_2$-NH$_2$ peptide. Accordingly, we synthesized these peptides and assessed the respective self-assembly properties in order to gain insight into the influence of peptide length on supramolecular assembly.
2.3.2 Analysis of peptide self-assembly.

All peptides were synthesized as the N-terminal acetyl, C-terminal amide sequences using standard Fmoc solid phase peptide synthesis methods on rink amide resin (see Experimental Section and Supporting Information (SI); Figures 2.8.S1–S6, Table 2.8.S1) for details of peptide synthesis, purification, and characterization.\textsuperscript{39} Truncation of the N-terminal Phe or the C-terminal Glu residue provides peptides that potentially have significantly different physicochemical properties from the parent Ac-(FKFE)\textsubscript{2}-NH\textsubscript{2} peptide due to the varying properties of the amino acids that are removed.\textsuperscript{40-42} For example, analytical HPLC analysis indicates that peptide hydrophobicity was altered by these subtle changes in length. Peptide hydrophobicity can be estimated by HPLC retention time: longer retention times on reverse phase stationary phases generally correspond to higher hydrophobicity.\textsuperscript{43} The parent peptide, Ac-(FKFE)\textsubscript{2}-NH\textsubscript{2}, and the C-terminal truncated Ac-FKFEFKF-NH\textsubscript{2} derivative have similar relative hydrophobicity based on identical retention times of 12.0 min (SI, Figures 2.8.S1-S2). The Ac-KFEFKFE-NH\textsubscript{2} peptide had a retention time of 11.3 min under identical mobile and stationary phase conditions (SI, Figure 2.8.S3), indicating that this peptide is significantly less hydrophobic than either the Ac-(FKFE)\textsubscript{2}-NH\textsubscript{2} or the Ac-FKFEFKF-NH\textsubscript{2} peptides. These observations illustrate that single amino acid changes in peptide length can dramatically influence the fundamental properties of the resulting peptides as a function of the properties of the truncated residues. Thus, changes in peptide self-assembly properties can also be anticipated.

Self-assembly of each peptide was initiated by dissolution of the lyophilized peptide in water. Self-assembly is concentration dependent as a function of peptide sequence
character so self-assembly was monitored at a low (0.2 mM) and a high (1.0 mM) concentration in order to ascertain whether the variants possessed differences in critical concentrations necessary to promote self-assembly. Peptide concentrations were verified by correlation to standard concentration curves constructed using amino acid analysis. The pH of the peptide solutions in water ranged from 3-4, presumably due to the presence of residual TFA from HPLC purification. Self-assembly was characterized using circular dichroism (CD) and Fourier transform infrared (FT-IR) spectroscopy, TEM imaging and electron diffraction analysis, and thermodynamic sedimentation analysis as described in the following sections.

2.3.3 Spectroscopic analysis of β-sheet self-assembly.

Amphipathic peptide self-assembly can be monitored by evolution of the requisite β-sheet secondary structure. CD and FT-IR spectroscopy are typically used in concert to confirm β-sheet secondary structure. Simple aqueous solutions can be monitored directly by CD spectroscopy, but FT-IR in the amide region I is sensitive to the presence of water and residual TFA. Therefore, IR analysis was preceded with a salt exchange by lyophilization of peptides from 1.0 % HCl in acetonitrile/water (40:60 v/v) to remove TFA counter-ions followed by isotopic exchange of water with D₂O through repeated lyophilization. CD and FT-IR spectra were recorded immediately after sample preparation to monitor formation of β-sheet secondary structure.

The parent Ac-(FKFE)₂-NH₂ and the N- and C-truncated peptides exhibited sequence-dependent effects on the formation of β-sheet secondary structure. The CD spectrum of the Ac-(FKFE)₂-NH₂ peptide corresponded to previously reported spectra, with a minima at 218 nm and 205 nm for both concentrations (0.2 mM and 1.0 mM) (Figure 2.3A). The
minimum at 218 nm is consistent with canonical β-sheet secondary structure,\textsuperscript{27,31,46-48} while the minimum at 205 nm has been previously attributed to either aromatic π-stacking\textsuperscript{49} effects in the hydrophobic core of the assembled β-sheet bilayer or to the formation of distorted β-sheet structures.\textsuperscript{50} The CD signal of the C-terminally truncated Ac-FKFEFKF-NH\textsubscript{2} peptide was similar to that of the parent sequence, with minima at 215 nm and 205 nm (Figure 2.3B). As for the parent peptide, this β-sheet secondary structure was observed at both concentrations (0.2 mM and 1.0 mM) for the Ac-FKFEFKF-NH\textsubscript{2} peptide. However, the N-terminal Phe-truncated peptide, Ac-KFEFKFE-NH\textsubscript{2}, exhibited dramatically different secondary structure. Its CD signal displayed a weak minimum at 200 nm at 0.2 mM (Figure 2.3C) that shifted to 205 nm at 1.0 mM. These spectra completely lacked a minimum at ~215 that would indicate formation of β-sheet structures. These spectra may be interpreted in several ways, but the most plausible interpretation is that the Ac-KFEFKFE-NH\textsubscript{2} peptide remains disordered (and therefore unassembled) under these conditions.\textsuperscript{31,47,51} It is possible that the reduced hydrophobicity of this sequence precludes its ability to assemble into β-sheet fibrils.
Figure 2.3. CD and FT-IR spectra for each peptide recorded in water or anion-exchanged D$_2$O for IR spectra (pH 3-4) at 1.0 mM (red) and 0.2 mM (blue). (A) Ac-(FKFE)$_2$-NH$_2$ CD spectra; (B) Ac-FKEFKF-NH$_2$ CD spectra; (C) Ac-KFEFKFE-NH$_2$ CD spectra. FT-IR spectrum (amide I region) at 1.0 mM for (D) Ac-(FKFE)$_2$-NH$_2$; (E) Ac-FKEFKF-NH$_2$; (F) Ac-KFEFKFE-NH$_2$.

FT-IR spectroscopy recorded in the amide I region was used to confirm the secondary structures since the reported CD spectra do not strictly conform to those of canonical β-sheet or disordered signals. The FT-IR spectrum of the Ac-(FKFE)$_2$-NH$_2$ peptide was consistent with previously reported spectra, exhibiting a strong signal at 1624 cm$^{-1}$ and a weaker signal at 1690 cm$^{-1}$ (Figure 2.3D). This spectrum is consistent with antiparallel β-sheet formation.$^{37,45,47,52}$ The FT-IR spectrum of the Ac-FKEFKF-NH$_2$ peptide (Figure 2.3E) was similar to that of the parent peptide, with a strong peak at 1620 cm$^{-1}$ and a weak signal at 1687 cm$^{-1}$. Based on similarity in both CD and IR spectra, the Ac-(FKFE)$_2$-NH$_2$ and Ac-FKEFKF-NH$_2$ peptides both appear to form structures that
feature antiparallel $\beta$-sheets. Lastly, the FT-IR spectrum for Ac-KFEFKFE-NH$_2$ exhibited a broad signal at 1645 cm$^{-1}$ (Figure 2.3F), confirming a disordered conformation for this peptide.$^{31,37,45}$ These findings are consistent with antiparallel $\beta$-sheet self-assembly for the Ac-(FKFE)$_2$-NH$_2$ and Ac-FKFEKF-NH$_2$ peptides, while the less hydrophobic Ac-KFEFKFE-NH$_2$ peptide remains disordered and, presumably, monomeric and unassembled.

2.3.4 Transmission electron microscopy (TEM) to confirm self-assembly and to characterize morphology of self-assembled structures.

CD and FT-IR spectroscopic analysis reports secondary structure but cannot independently confirm formation of highly ordered self-assembled structures. High-resolution microscopic imaging analyses to confirm that $\beta$-sheet secondary structures observed in spectroscopic analyses correspond to formation of self-assembled materials. We, therefore, conducted TEM analyses of each peptide at both concentrations in order to assess self-assembly and to characterize the morphology of the self-assembled structures. The Ac-KFEFKFE-NH$_2$ peptide lacked higher order structures in all TEM images, consistent with spectroscopic data that suggests that this peptide is un assembled (images not shown). Interestingly, TEM images of the Ac-(FKFE)$_2$-NH$_2$ and Ac-FKFEKF-NH$_2$ peptides showed distinct morphological differences between the assembled structures. As previously reported, characteristic left-handed helical nanoribbons were observed for Ac-(FKFE)$_2$-NH$_2$ at both 1.0 mM and 0.2 mM concentrations (Figure 2.4A-B). The nanoribbon widths were $8.2 \pm 1.2$ nm and $8.1 \pm 0.9$ nm and had a helical pitch of $18.2 \pm 1.5$ nm and $18.2 \pm 1.6$ nm at 1.0 mM and 0.2 mM concentrations, respectively (Table 2.1). The nanoribbons were micrometers in length.$^{31,35,36,46,47}$ The helical coiled structures are presumed to relax over time into flat nanoribbon structures with the same widths as
were observed in the helical materials. These observations are consistent with previously reported data for the Ac-(FKFE)$_2$-NH$_2$ peptide.$^{31,35,36}$

Figure 2.4. TEM images of Ac-(FKFE)$_2$-NH$_2$ nanoribbons at (A) 1.0 mM and (B) 0.2 mM concentrations; TEM images of Ac-FKFEFKF-NH$_2$ nanotapes and fibrils at (C) 1.0 mM and (D) 0.2 mM concentrations.
Despite nearly identical CD and IR spectra relative to the parent Ac-(FKFE)$_2$-NH$_2$ peptide, the Ac-FKFEFKF-NH$_2$ peptide self-assembled into materials of distinct morphology. Mixtures of two unique morphologies were observed for the Ac-FKFEFKF-NH$_2$ peptide at both 1.0 mM and 0.2 mM concentrations (Figure 2.4C-D). Broad, gently twisting nanotapes with widths of 19.2 ± 3.8 nm and 20.1 ± 3.1 nm at 1.0 mM and 0.2 mM, respectively were found to be mixed with thinner fibrils of 3.7 ± 0.7 nm (1.0 mM) and 4.0 ± 0.8 nm (0.2 mM) widths (Table 2.1). However, similar to Ac-(FKFE)$_2$-NH$_2$ both the nanotapes and fibrils were micrometers in length.

<table>
<thead>
<tr>
<th>Table 2.1. Compiled dimensions of self-assembled materials derived from sequence altered amphipathic peptides at varying concentrations and pH.</th>
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<tbody>
<tr>
<td>Ac-(FKFE)$_2$-NH$_2$ Fibril Width$^a$ (nm)</td>
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<tr>
<td>pH 4 1.0 mM</td>
</tr>
<tr>
<td>pH 4 0.2 mM</td>
</tr>
<tr>
<td>pH 7 1.0 mM</td>
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<td>pH 7 0.2 mM</td>
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$^a$ Helical nanoribbons  
$^b$ Broad nanotapes

Electron diffraction patterns were obtained using TEM instrumentation in order to confirm $\beta$-sheet structure via strand spacings of ~4.7 Å. Calculated $d$-spacing values for Ac-(FKFE)$_2$-NH$_2$ fibrils were derived from the electron diffraction pattern and were found to be 4.7 Å at both 1.0 mM and 0.2 mM concentrations (Figure 2.5 and SI, Figure 2.8.S7).$^{37,38,53}$ The Ac-FKFEFKF-NH$_2$ fibrils had a slightly smaller $d$-spacing of 4.5 Å (Figure 2.5 and SI, Figure 2.8.S7). The shorter $d$-spacing for Ac-FKFEFKF-NH$_2$ fibrils is within error and does not likely indicate a significant deviation in packing pattern within
the β-sheet fibrils. Thus, the differences in fibril morphology most likely arise from differences in interactions between β-sheets at the edges.

**Figure 2.5.** Electron diffraction patterns for Ac-(FKFE)$_2$-NH$_2$ fibrils at 1.0 mM (red) and 0.2 mM (blue) concentrations (4.7 Å) and Ac-FKF-FKF-NH$_2$ fibrils at 1.0 mM (green) and 0.2 mM (black) concentrations (4.5 Å).

The dramatically different assembled structures formed by the Ac-(FKFE)$_2$-NH$_2$ and Ac-FKF-FKF-NH$_2$ peptides were unanticipated and illustrate that minor changes in peptide sequence can have significant effects on peptide self-assembly. While spectroscopic data suggests that both the Ac-(FKFE)$_2$-NH$_2$ and Ac-FKF-FKF-NH$_2$ peptides adopt antiparallel β-sheet structures, the ability of the latter peptide to adopt in-
register sheet structures provides assembled materials with unique properties. The helical nanoribbons formed by Ac-(FKFE)$_2$-NH$_2$ have widths of ~8 nm. This is consistent with the side-by-side arrangement of two $\beta$-sheets within the nanoribbon structure (Figure 2.6A).$^{23,54}$ Conversely, the 3.7 nm fibrils formed by the Ac-FKFEFKF-NH$_2$ peptide are consistent with $\beta$-sheet bilayers that are a single peptide wide (Figure 2.6B). The much wider ~20 nm wide nanotapes formed by the Ac-FKFEFKF-NH$_2$ peptide must be composed of at least 5 side-by-side $\beta$-sheets. Perhaps the “blunt” edges of these sheets that are putative in-register, antiparallel $\beta$-sheets lacking unpaired amino acids can more readily align edge-to-edge to form wider nanotapes that are possibly composed of multiple aligned 3.7 nm fibrils that represent the fundamental $\beta$-sheet unit (Figure 2.6B). However, a staggered strand alignment is possible but requires specific patterning between $\beta$-sheets for fibrils widths equivalent to 5 side-by-side laminations thus causing this registry to be less likely (Figure 2.6C).$^{24,55}$ Ideally, each peptide could assemble infinity side-by-side in either direction but thermodynamics halt lamination at specific fibril widths. The dangling $N$-terminal Phe of the Ac-(FKFE)$_2$-NH$_2$ fibrils may perturb the geometry of edge-to-edge alignment of the basic $\beta$-sheet bilayer structures, effectively limiting nanoribbon width to two $\beta$-sheets. However, the “blunt” edges result in fibrils widths of approximately five laminations for Ac-FKFEFKF-NH$_2$.$^{56}$ Additional studies are necessary to fully understand these unexpected differences in fibril structure.
Figure 2.6. Possible strand alignments for Ac-(FKFE)$_2$-NH$_2$ and Ac-FKFEFKF-NH$_2$ that highlight electrostatic interactions (rectangles) along with their β-strand (Top) and fibril (Bottom) dimensions. (A) Out-of-register cross-strand pairing for Ac-(FKFE)$_2$-NH$_2$ with complementary electrostatic interactions has a β-strand width of ~3-4 nm that causes two strands to laminate into β-sheets with widths of ~8-9 nm; (B) In-register cross-strand pairing for Ac-FKFEFKF-NH$_2$ has potential unfavorable electrostatic interactions with a β-strand width of ~3-4 nm that causes multiple strands to laminate into β-sheets with widths from ~18-24 nm; (C) Staggered cross-strand pairing for Ac-FKFEFKF-NH$_2$ with complementary electrostatic interactions is less likely, but multiple β-strands with a width of ~3-4 nm could laminate side-by-side into β-sheets with widths of from ~18-24 nm.
2.3.5 Self-assembly at neutral pH.

Self-assembly of amphipathic peptides in which the hydrophilic groups possess ionizable side chains is sensitive to pH\(^{57-59}\). In the self-assembly studies reported herein, the peptides are dissolved in water at pH 3–4 as a function of residual acids from peptide purification\((16,31)\). Under these conditions each of the three peptides most likely displays protonated Glu side chains (neutral) and Lys side chains (positive), giving peptides with an overall charge of +2. As modeled in Figure 2.2, possible attractive charge interactions between Glu and Lys side chains in the putative Ac-(FKFE)\(_2\)-NH\(_2\) and Ac-KFEFKFE-NH\(_2\) \(\beta\)-sheet structures are not exploited. The Ac-FKFEFKF-NH\(_2\) \(\beta\)-sheets would feature repulsive effects between neighboring charged Lys residue across strand. At neutral pH, Glu side chains are deprotonated (negative) and Lys side chains remain protonated (positive). Thus, Ac-(FKFE)\(_2\)-NH\(_2\) and Ac-KFEFKFE-NH\(_2\) would be neutral and possess possible attractive charge interactions between charged residues at pH 7. The Ac-FKFEFKF-NH\(_2\) would have a charge of +1 and possess possible repulsive interactions between all hydrophilic side chains. We reasoned, based on this analysis, that self-assembly may be dramatically different at pH 7 than at pH 3–4. Therefore, we assessed the self-assembly of each sequence in phosphate buffer at pH 7 as a comparison to studies conducted in water (pH 3).

Self-assembly of all three peptides was characterized by dissolution at 1.0 mM and 0.2 mM concentrations in phosphate buffer (pH 7). While these peptides, even in an assembled state, were soluble in water (pH 3–4), it was found that at neutral pH precipitation was observed. This is most likely due to the reduced overall charge state of the peptides/fibrils at neutral pH. This lower overall charge state also reduces the surface
charge of the exposed hydrophilic faces of the $\beta$-sheet bilayer structures; thus fibril-fibril interactions are more likely and bundling of self-assembled fibrils/nanoribbons/nanotapes into higher order aggregates with reduced water solubility may also occur. Precipitation made CD analysis problematic due to light scattering effects. We, therefore, limited our analysis of self-assembly at neutral pH to TEM analysis of suspended precipitates.

TEM images indicated that self-assembly occurs for all peptides at neutral pH (Figure 2.7). The Ac-(FKFE)$_2$-NH$_2$ peptide formed nanoribbons at both concentrations (Figure 2.7A-B); at neutral pH, however, fewer helical nanoribbons were observed, with most of the nanoribbons existing in relaxed flattened states. The Ac-(FKFE)$_2$-NH$_2$ nanoribbons at neutral pH were slightly wider than at pH 3-4, measuring 10.3 $\pm$ 2.3 nm and 10.9 $\pm$ 3.3 nm at 1.0 mM and 0.2 mM, respectively (Table 2.1). For Ac-FKFEFKF-NH$_2$, nanotapes of 10.9 $\pm$ 3.3 nm and 10.9 $\pm$ 3.4 width were observed, but these were found to be extensively aggregated into bundled structures (Figure 2.7C-D). Lastly, pH had the greatest effect on Ac-KFEFKFE-NH$_2$ (which was unassembled at pH 3–4), promoting self-assembly into flat nanotapes of 10.9 $\pm$ 3.6 nm and 10.9 $\pm$ 3.7 widths similar to the other assembled peptides; Ac-KFEFKFE-NH$_2$ also bundled into higher order aggregate structures (Figure 2.7E-F). It is interesting that only the seven-amino acid peptides formed bundled nanotapes, whereas the eight amino acid peptide maintained narrow 10 nm nanoribbons. The electron diffraction patterns for all peptides had $d$-spacings of $\sim$4.7 Å indicating $\beta$-sheet formation (SI, Figure 2.8.S8-S9).
Figure 2.7. TEM images of fibrils in phosphate buffer (pH 7). Ac-(FKFE)$_2$-NH$_2$ fibrils at (A) 1.0 mM (B) 0.2 mM; Ac-FKFEFKF-NH$_2$ fibrils at (C) 1.0 mM (D) 0.2 mM; Ac-KFEFKFE-NH$_2$ fibrils at (E) 1.0 mM (F) 0.2 mM.
2.3.6 Thermodynamic effects of sequence length on peptide self-assembly.

Self-assembling peptides ultimately reach an equilibrium state that can be represented in a simplified form as $\text{fibril}_n + \text{monomer} \leftrightarrow \text{fibril}_{n+1}$. This end-state equilibrium, which involves the association/dissociation of one molecule of peptide to the fibril end, is characteristic of any given peptide as a function of self-assembly conditions. Thus, measurement of monomer concentration at equilibrium (defined as the critical concentration, $C_r$, below which self-assembly of the peptide does not occur) can allow comparative analysis of the relative thermodynamic favorability for self-assembly of similar peptides. Accordingly, we conducted studies to understand possible thermodynamic effects of sequence length on the self-assembly of each peptide reported herein.

Sedimentation analysis was used to interrogate the relative equilibrium between monomer and fibril for each peptide. This was accomplished by modifying Wetzel’s HPLC sedimentation assay that has been used to study thermodynamic effects in amyloid peptide self-assembly. Wetzel’s assay relies on measurement of monomer concentration at equilibrium ($C_r$) in peptide self-assembly processes in order to understand the thermodynamic effects that amino acid substitutions have on self-assembly. While $C_r$ values can be used to infer relative free energy differences ($\Delta G$) for self-assembly of related peptides, these $\Delta G$ values are reliable only when the fibrils compared have identical peptide packing modes within the assembled fibrils. For comparison of fibrils in which dramatically different morphologies are observed for the self-assembled materials, it must be assumed that a significant difference in peptide packing is possible/probable, thus making calculated comparative $\Delta G$ values from measured $C_r$ values difficult to
interpret. Thus, we report only equilibrium $C_r$ values for each of the peptides studies herein. While comparative $\Delta G$ values cannot be reliably reported, equilibrium $C_r$ values still allow a rudimentary assessment of the thermodynamic effects of the changes in peptide length for the reported sequences. Therefore, a lower $C_r$ value can be assumed to correspond to a thermodynamic advantage for self-assembly for a given peptide.

Sedimentation analysis was conducted on each peptide at both reported self-assembly concentrations (0.2 mM and 1.0 mM). Briefly, each peptide was incubated in water as described previously and self-assembly was allowed to proceed to equilibrium (defined as the point at which monomer concentration stabilizes). Monomer concentration ($C_r$) was measured by sedimentation of fibril and other aggregate material via centrifugation and measurement of monomer concentration in the supernatant by injection onto an HPLC instrument and correlation of the integrated peak area for each peptide to an established concentration curve (see Experimental Section and SI, Figure 2.8.S10-11). Each peptide was also incubated at identical concentrations in acetonitrile/water, which prevents peptide self-assembly; these control solutions were subjected to sedimentation conditions in order to confirm that monomer concentration does not change and that starting peptide concentrations were accurate.

$C_r$ values for each peptide are compiled in Table 2.2. $C_r$ values for self-assembly of the Ac-(FKFE)$_2$-NH$_2$ peptide were 59 ± 4 \mu M and 53 ± 3 \mu M at starting concentrations of 0.2 mM and 0.1 mM, respectively. As expected, the values for $C_r$ were nearly identical at both starting concentrations since $C_r$ is an inherent characteristic of a given self-assembling peptide dependent on solvent, temperature, and pH.$^{52}$ The Ac-FKFEFKF-NH$_2$ peptide had moderately higher $C_r$ values of 70 ± 3 \mu M and 63 ± 2 \mu M at 0.2 mM and 1.0
mM initial peptide concentrations, respectively. These values suggest that truncation of the C-terminal Glu residue of the parent peptide results in a sequence that has slightly lower propensity to self-assemble. Since the parent Ac-(FKFE)₂-NH₂ peptide and the Ac-FKFEEKF-NH₂ peptide have similar hydrophobicity, these modest differences in \( C_r \) may be due to a slight increase in electrostatic repulsion between Lys side chains paired cross-strand in the hypothetical antiparallel \( \beta \)-sheet packing structure of the latter sequence (Figure 2.2B). However, these differences in \( C_r \) could also arise from altered packing effects for the self-assembled Ac-FKFEEKF-NH₂ nanotape morphology relative to the Ac-(FKFE)₂-NH₂ nanoribbons (Figure 2.6). Finally, \( C_r \) values for the Ac-KFEEKFE-NH₂ peptide were \( 218 \pm 10 \) µM and \( 975 \pm 70 \) µM at 0.2 mM and 1.0 mM starting concentrations, respectively. These values confirm that the Ac-KFEEKFE-NH₂ peptide is monomeric and unassembled under these conditions. Based on HPLC trace analysis, Ac-KFEEKFE-NH₂ is significantly less hydrophobic than either Ac-(FKFE)₂-NH₂ or Ac-FKFEEKF-NH₂, thus explaining its dramatically lower propensity to self-assemble under these conditions (Figure 2.8.S1-3).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>( C_r ) (µM) (0.2 mM peptide)</th>
<th>( C_r ) (µM) (1.0 mM peptide)</th>
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<tbody>
<tr>
<td>Ac-(FKFE)₂-NH₂</td>
<td>59 ± 4</td>
<td>53 ± 3</td>
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<tr>
<td>Ac-FKFEEKF-NH₂</td>
<td>70 ± 3</td>
<td>63 ± 2</td>
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<tr>
<td>Ac-KFEEKFE-NH₂</td>
<td>218 ± 10</td>
<td>975 ± 70</td>
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</table>
2.4 Discussion

Subtle changes in sequence length of amphipathic peptides can thus have dramatic effects on the thermodynamic propensity to undergo self-assembly. Observed differences for truncated variants of the parent Ac-(FKFE)$_2$-NH$_2$ peptide can be primarily attributed to two factors. First, removal of a single amino acid can have dramatic effects on the physicochemical properties of the resulting peptide. Truncation of the N-terminal Phe residue gives a peptide, Ac-KFEFKFE-NH$_2$ that is significantly less hydrophobic that the parent peptide. Peptide hydrophobicity is a major determinant of self-assembly propensity.$^{31,47,52,61,63,65,66}$ Second, changes in length can also perturb packing architecture in several ways. Removal of the C-terminal Glu residue results in a putative cross-strand packing structure in which like charges are paired (Figure 2.2B), resulting in subtle destabilization of the resulting $\beta$-sheet. In addition, the unique morphology of the Ac-FKFEFKF-NH$_2$ peptide nanotapes relative to the parent nanoribbons is most likely due to changes in $\beta$-sheet edge-to-edge packing. This may arise from fundamental $\beta$-sheet units that have exclusively hydrophobic edges due to flanking Phe residues at both edges, whereas the fundamental sheet of Ac-(FKFE)$_2$-NH$_2$ peptides has a hydrophilic Glu that perturbs hydrophobicity at the sheet edge. These results demonstrate that subtle changes in sequence length of amphipathic peptides can result in complex thermodynamic effects in self-assembly of the resulting sequences.
2.5 Conclusion

Amphipathic peptide self-assembly is sensitive to subtle changes in sequence length. Herein, we have reported that truncation of single amino acid from either the $N$- or $C$-terminus of the amphipathic Ac-(FKFE)$_2$-NH$_2$ peptide results in sequences with dramatically different self-assembly behavior. Truncation of the $N$-terminal Phe residue gives a peptide, Ac-KFEFKFE-NH$_2$ that fails to self-assemble in water at acidic pH (pH 3–4); self-assembly is only observed at neutral pH, which enforces a neutral overall peptide charge that increases peptide hydrophobicity and optimizes cross-strand charge pairing at the hydrophilic face of the $\beta$-sheet. Conversely, truncation of the $C$-terminal Glu residue provides a sequence, Ac-FKFEFKF-NH$_2$ that readily self-assembles, albeit into fibrils with unique broad nanotape morphology relative to the twisted nanoribbons of the parent Ac-(FKFE)$_2$-NH$_2$ peptide. Interestingly, self-assembly of the Ac-KFEFKFE-NH$_2$ peptide at neutral pH also formed broad nanotape structures, suggesting that this morphology may be a general feature of odd-numbered amphipathic peptide sequences. Future studies will focus on the possible effects of these varying morphologies on the emergent properties of the resulting materials, including hydrogelation. These studies provide significant insight into the effects of sequence length on the self-assembly of amphipathic peptides and indicate that subtle changes in length can facilitate tuning of peptide self-assembly processes for the design of next-generation materials derived from self-assembled peptides.
2.6 Experimental Section

2.6.1 Peptide synthesis.

Peptides were synthesized using standard Fmoc protection and HBTU/HOBt activation strategies on a microwave-equipped Liberty Peptide Synthesizer (CEM®). Rink amide resin (Advanced ChemTech, 100-200 mesh, 0.2 mmol g\(^{-1}\)) was utilized to provide C-terminal amide peptides; resin was loaded by HBTU/HOBt activation protocols. Peptides were prepared with N-terminal acetyl groups; cleavage from the solid support and side chain deprotection was accomplished by treatment with trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water (95:2.5:2.5, v/v) (room temperature, 1 h). The cleavage mixture was concentrated to 10% of the reaction volume and the peptide was then precipitated by addition to cold ethyl ether and isolated by centrifugation; the resulting peptide was washed once by resuspension in cold ether. The resulting material was dissolved in DMSO prior to purification by high-pressure liquid chromatography (HPLC).

2.6.2 Peptide purification and characterization.

Purification of synthetic peptides was conducted by (HPLC) using a Shimadzu LC-AD HPLC instrument with a reverse phase C18 column (Waters, BEH300, 10 µm, 19 × 250 mm). A binary gradient of acetonitrile and water with 0.1% TFA at 10 mL min\(^{-1}\) was used and eluent was monitored by UV absorbance at 215 and 254 nm. Fractions were collected and purity assessed by analytical HPLC on a reverse phase C18 column (Waters, BEH300, 10 µm, 4.6 × 250 mm). Peptide identity was confirmed by MALDI-TOF mass spectroscopic analysis: (Ac-FKFEFKE-NH\(_2\)) \(m/z\) 1162.35 (1162.34 calcd for [MH]\(^+\)), \(m/z\) 1185.46 (1185.33 calcd for [MNa]\(^+\)), \(m/z\) 1201.92 (1201.44 calcd for
[MK]⁺; (Ac-KFEFKFE-NH₂) m/z 1015.80 (1015.53 calcd for [MH]⁺), m/z 1037.75 (1037.51 calcd for [MNa]⁺), m/z 1053.70 (1053.65 calcd for [MK]⁺); (Ac-FKFEFKF-NH₂) m/z 1033.72 (1033.55 calcd for [MH]⁺), m/z 1055.70 (1055.53 calcd for [MNa]⁺), m/z 1071.68 (1071.67 calcd for [MK]⁺) (See Table 2.8.S1 and Figure 2.8.S1-6 in Supporting Information for HPLC and MALDI-MS data).

2.6.3 Peptide self-assembly.

Peptide self-assembly was analyzed in water and in phosphate buffered solution (pH 7). Peptide solutions in unbuffered water had pH values from 3–4 presumably due to residual TFA from HPLC purification of the respective peptides. Self-assembly was analyzed at peptide concentrations of 1.0 mM and 0.2 mM. Peptide concentration was quantified as previously described.

Briefly, each peptide was dissolved in acetonitrile/water (mixed organic/aqueous solutions maintain an unassembled state of the peptide) and a sample of each was assessed by analytical HPLC; peptide concentration was determined by correlation of HPLC peak area to an HPLC standard curve constructed for each peptide (see Figures 2.8.S10-11 for standard curves). The absolute concentration for each HPLC standard curve was determined by amino acid analysis (AIBiotech, Richmond, VA). Once concentration was determined, each peptide was separated into aliquots of the desired quantity and these samples were subsequently frozen and lyophilized. Self-assembly was initiated by dissolving lyophilized peptides in water (1.0 mM and 0.2 mM concentrations). Optically transparent, homogenous solutions were obtained after three cycles of vortex (1 min) and sonication (5 min). Self-assembly was characterized by circular dichroism (CD) and Fourier transform infrared (FT-IR)
spectroscopy correlated to transmission electron micrograph (TEM) images as described in the following sections.

2.6.4 **Circular dichroism (CD) spectroscopy.**

CD spectra were recorded on an AVIV 202 circular dichroism spectrometer. Spectra were obtained using a 0.1 mm path length quartz cuvette (Hellma) at 25 °C from 260 to 190 nm with a 1.0 nm step, 1.0 nm bandwidth, and a 3 s collection time per step. Background subtraction, conversion to molar ellipticity, and data smoothing with a least squares fit was performed using the AVIV software.

2.6.5 **Fourier transform infrared (FT-IR) spectroscopy.**

FT-IR spectra were obtained using a Shimadzu 8400 FT-IR spectrometer. Anion exchange to remove TFA counter-ions from purified peptides was performed prior to FT-IR analysis; anion exchanged was performed by dissolving peptides in 40% acetonitrile and water (v/v) with 1% HCl followed by lyophilization. The freeze and lyophilization process was repeated twice in 40% acetonitrile/D$_2$O. Each peptide was then dissolved in D$_2$O (1 mM) followed by three cycles of vortex (1 min) and sonication (5 min). An aliquot of each peptide solution (70 µL) was analyzed using 25 × 4 mm CaF$_2$ plates (International Crystal Labs); measurements were obtained from 1550 to 1750 cm$^{-1}$ using the Happ-Genzel method and 1024 scans.

2.6.6 **Negative-stain transmission electron microscopy (TEM) imaging and electron diffraction.**

Confirmation of peptide self-assembly and characterization of the morphology of the assembled material was accomplished by TEM analysis. An aliquot of each assembled peptide solution (10 µL) was applied to 200-mesh carbon-coated copper grids. After standing for 2 min, excess fluid was removed by capillary action. Peptide fibrils were
then stained by applying 10 µL of 5% uranyl acetate to the grid for 1 min; remaining solvent was removed by capillary action and the grids were allowed to dry prior to imaging. For 1.0 mM fibril samples, the self-assembly solutions were diluted five-fold prior to application to the grid in order to reduce fibril concentration. Electron micrograph images were obtained using an Hitachi 7650 transmission electron microscope in high-contrast mode at an accelerating voltage of 80 kV. Fibril dimensions were measured in ImageJ obtained from the NIH website (http://rsbweb.nih.gov/ij/); reported dimensions are the average of at least 100 measurements on distinct fibrils for each self-assembled peptide. Electron diffraction experiments were also performed on each sample using a spot size of 30 nm and a camera length of 2 m. Diffraction profiles were obtained using the program ImageJ and fibril $d$-spacing values were calculated from the diffraction profile using Bragg’s Law (Figure 2.5 and SI Figure 2.8.S7-S9).\textsuperscript{37,62}

2.6.7 Sedimentation analysis.

Sedimentation analysis was used to interrogate the relative equilibrium between monomer and fibril for each peptide. This was accomplished by modifying Wetzel’s HPLC sedimentation assay that has been used to study the thermodynamic effects of substitutions on amyloid self-assembly.\textsuperscript{64} Each peptide (Ac-(FKFE)$_2$-NH$_2$, Ac-FKFEFKF-NH$_2$, Ac-KFEFKFE-NH$_2$) was allowed to self-assemble at 1.0 mM or 0.2 mM concentrations as described in the peptide self-assembly section above. Control experiments were prepared at identical concentrations in 60% acetonitrile/water (v/v) with 0.1% TFA; self-assembly does not occur under these solution conditions allowing comparative assessment of peptide solutions that maintain a monomeric state. Monomer concentration at equilibrium (critical concentration, $C_r$) was determined as a function of
time by centrifuging samples (100,000 g, 1 h, 4 ºC) in order to sediment fibrils and other higher order aggregates. Samples in both water (assembled) and acetonitrile/water (unassembled) were treated identically. Aliquots (0.1 mL) were carefully removed from the supernatant and diluted 20 fold into DMSO. It should be noted that these aliquots were removed from the upper one-third volume of centrifuged samples because the sedimented fibril material remains largely soluble even after centrifugation; a visible pellet is often not observed. Tubes were handled with great care after centrifugation to avoid resuspension of the sedimented material. The concentration of monomer ($C_r$) in each sample was determined by injection of DMSO supernatant solutions onto an analytical HPLC instrument and correlation of integrated peak area to a standard curve as described in the peptide self-assembly section. Experiments were repeated in triplicate and error in $C_r$ measurement is reported as the standard deviation about the mean.
2.7 References


2.8 APPENDIX: Supporting information and data

Figure 2.8.S1. Analytical HPLC trace (215nm) for Ac-(FKFE)$_2$-NH$_2$.

Figure 2.8.S2. Analytical HPLC trace (215nm) for Ac-FKF-EFKF-NH$_2$.

Figure 2.8.S3. Analytical HPLC trace (215nm) for Ac-KFEFKF-NH$_2$. 
Table 2.8.S1. Calculate and observed m/z for synthetic peptides (MALDI-TOF-MS).

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Figure 2.8.S4. MALDI-TOF-MS data for Ac-(FKFE)_2-NH₂.
Figure 2.8.S5. MALDI-TOF-MS data for Ac-FKFEFKF-NH₂.

Figure 2.8.S6 MALDI-TOF-MS data for Ac-KFEFKFE-NH₂.
Figure 2.8.S7. Electron diffraction in water (pH 3-4). (A) Ac-(FKFE)$_2$-NH$_2$ at 1.0 mM; (B) Ac-(FKFE)$_2$-NH$_2$ at 0.2 mM; (C) Ac-FKFEFKF-NH$_2$ at 1.0 mM; (D) Ac-FKFEFKF-NH$_2$ at 0.2 mM.
Figure 2.8.S8. Electron diffraction in phosphate buffer (pH 7). (A) Ac-(FKFE)$_2$-NH$_2$ at 1.0 mM; (B) Ac-(FKFE)$_2$-NH$_2$ at 0.2 mM; (C) Ac-FKFEFKF-NH$_2$ at 1.0 mM; (D) Ac-FKFEFKF-NH$_2$ at 0.2 mM; (E) Ac-KFEFKFE-NH$_2$ at 1.0 mM; (F) Ac-KFEFKFE-NH$_2$ at 0.2 mM.
Figure 2.8.S9. Electron diffraction patterns in phosphate buffer (pH 7) for Ac-(FKFE)$_2$-NH$_2$, Ac-FKFEKF-NH$_2$, and Ac-KFEFKFE-NH$_2$ at 1.0 mM and 0.2 mM.
Figure 2.8.S10. HPLC calibration curve (215 nm) for Ac-FKFEKFE-NH$_2$.

Figure 2.8.S11. HPLC calibration curve (215 nm) for Ac-KFEFKFE-NH$_2$. 
Chapter 3

3 Effects of Varied Sequence Pattern on the Self-Assembly of Amphipathic Peptides

3.1 Abstract

Amphipathic peptides have an increased propensity to self-assemble into amyloid-like \( \beta \)-sheet fibrils when their primary sequence pattern consists of alternating hydrophobic and hydrophilic amino acids. These fibrils adopt a bilayer architecture composed of two \( \beta \)-sheets laminated to bury the hydrophobic side chains of the \( \beta \)-sheet in the bilayer interior, leaving the hydrophilic side chains exposed at the bilayer surface. In this study, the effects of altering the sequence pattern of amphipathic peptides from
strictly alternating hydrophobic/hydrophilic repeats to more complex patterning of hydrophobic and hydrophilic residues on self-assembly of the resulting sequences are reported. Self-assembly of the Ac-(FKFE)$_2$-NH$_2$ peptide was compared to that of four related sequences with varied amino acid sequence patterning: Ac-(FK)$_2$(FE)$_2$-NH$_2$, Ac-KEFFFKE-NH$_2$, Ac-(KFFE)$_2$-NH$_2$, and Ac-FFKEKEFF-NH$_2$. The Ac-(FKFE)$_2$-NH$_2$ and Ac-(FK)$_2$(FE)$_2$-NH$_2$ peptides effectively self-assemble at high (1.0 mM) and low (0.2 mM) concentrations (pH 3–4) into β-sheet nanoribbons that were 8 nm and 4 nm wide, respectively. The Ac-KEFFFKE-NH$_2$ peptide failed to self-assemble at low concentration (pH 3–4), but self-assembled into distinct nanotapes that were ~20 nm in width at high concentration. Ac-(KFFE)$_2$-NH$_2$ and Ac-FFKEKEFF-NH$_2$ failed to self-assemble into fibril/tape-like materials at either high or low concentration at pH 3–4, although Ac-FFKEKEFF-NH$_2$ formed micelle-like aggregates at higher concentrations.

At neutral pH, similar self-assembly behavior was observed for each peptide as was observed at acidic pH. An exception was the Ac-FFKEKEFF-NH$_2$ peptide, which formed ~20 nm nanotapes at neutral pH. These results indicate that amino acid sequence patterns exert a profound influence on self-assembly propensity and morphology of the resulting materials even when the overall hydrophobicity or charge of the related peptides are identical. Sequence pattern variation can thus be exploited as a variable in the creation of novel materials composed of self-assembled peptides.

### 3.2 Introduction

Peptide and protein self-assembly into β-sheet fibril structures has been a subject of significant recent interest. This is due, in part, to the association of aberrant self-assembly of proteins into cross-β amyloid materials with a growing list of pathologies that include
Alzheimer’s disease, Creutzfeldt-Jakob disease, and type II diabetes.\textsuperscript{1-4} Cross-\textit{\beta} amyloid also exists as a conserved, functional motif in many organisms.\textsuperscript{5,6} As such, there have been efforts to exploit the self-assembly of \textit{\beta}-sheet peptides and proteins in the creation of functional biomaterials. Biomaterials derived from amyloid-inspired self-assembled peptides have been exploited as hydrogel matrices for tissue engineering,\textsuperscript{7,8} scaffolds for vaccine development,\textsuperscript{9-12} drug delivery vectors,\textsuperscript{13-16} and numerous other inventive applications.\textsuperscript{17-19} Despite the great success in the application of self-assembled \textit{\beta}-sheet materials, fundamental questions regarding the mechanisms of self-assembly and the relationship of these mechanisms to the emergent properties of the resulting materials persist, impeding rational design of next-generation materials with precisely tuned properties.

Amphipathic peptides have an increased propensity to self-assemble into amyloid-like \textit{\beta}-sheet fibrils when their primary sequence pattern consists of alternating hydrophobic and hydrophilic amino acids (Figure 3.1A).\textsuperscript{20-23} The sequence pattern of alternating polar and nonpolar residues in a generic (XZXZ)\textsubscript{n} motif (X is a nonpolar and Z is a polar amino acid) provides sequences that have been shown to assemble into \textit{\beta}-sheets in which the hydrophilic side chains and hydrophobic side chains are segregated to opposite faces of the sheet. Lamination of these \textit{\beta}-sheets into putative bilayer structures results in burial of the hydrophobic (X) side chain groups within a hydrophobic core that exposes the hydrophilic (Z) functionality to the aqueous solvent (Figure 3.1B). The assembled fibrils are thus highly water-soluble. This unique characteristic has facilitated exploitation of the resulting \textit{\beta}-sheet fibrils in a wide range of diverse applications.
Figure 3.1. (A) Ac-(FKFE)$_2$-NH$_2$, an example of an amphipathic peptide with alternating hydrophobic and hydrophilic amino acids that efficiently self-assembles into β-sheet fibrils. (B) Schematic representation of the putative β-sheet bilayer structure formed by Ac-(FKFE)$_2$-NH$_2$ self-assembly.

Most naturally occurring amyloid peptides are amphipathic sequences, but lack a strict pattern of alternating hydrophobic and hydrophilic residues. Thus, while the
alternating \((XZXZ)_n\) patterned peptides assemble into \(\beta\)-sheet fibrils with interesting emergent properties, the role of this sequence pattern on peptide self-assembly propensity has not been carefully assessed. In this study, the effects of altering the sequence pattern of amphipathic peptides from strictly alternating hydrophobic/hydrophilic repeats to more complex patterning of hydrophobic and hydrophilic residues on self-assembly of the resulting sequences is reported. We used the frequently studied Ac-(FKFE)\(_2\)-NH\(_2\) peptide as a model for this analysis. Self-assembly of the Ac-(FKFE)\(_2\)-NH\(_2\) peptide was compared to that of four related sequences with varied amino acid sequence patterning: Ac-(FK)\(_2\)(FE)\(_2\)-NH\(_2\), Ac-KEFFFKE-NH\(_2\), Ac-(KFFE)\(_2\)-NH\(_2\), and Ac-FFKEKEFF-NH\(_2\). It is significant that these peptides are identical in terms of length, amino acid composition, hydrophobicity, and charge; they differ only in terms of sequence pattern.

### 3.2.1 Experimental rationale and peptide design.

In order to characterize the effects of sequence patterning on the self-assembly of amphipathic \(\beta\)-sheet peptides, we conducted a study comparing self-assembly of the Ac-(FKFE)\(_2\)-NH\(_2\) peptide with variants featuring altered amino acid sequence patterns. The parent Ac-(FKFE)\(_2\)-NH\(_2\) peptide readily self-assembles into \(\beta\)-sheet bilayers (Figure 3.1) and has been frequently used as a model for peptide self-assembly and as a functional material.\(^{21-29}\) For the studies reported herein, the primary Ac-(FKFE)\(_2\)-NH\(_2\) sequence was rearranged into four unique sequences: Ac-(FK)\(_2\)(FE)\(_2\)-NH\(_2\), Ac-KEFFFKE-NH\(_2\), Ac-(KFFE)\(_2\)-NH\(_2\), and Ac-FFKEKEFF-NH\(_2\). The Ac-(FKFE)\(_2\)-NH\(_2\) and Ac-(FK)\(_2\)(FE)\(_2\)-NH\(_2\) peptides were the only variants that maintained the alternating pattern of hydrophobic and hydrophilic residues. Gazit, Serpell, and others have shown that the Phe-Phe dipeptide motif and peptides containing this motif are capable of self-assembly.\(^{30-33}\) Therefore, the
other three derivatives were designed with variably spaced Phe-Phe motifs separated by 0, 1, or 2 pairs of complementary hydrophilic residues (Ac-KEFFFFKE-NH$_2$, Ac-(KFFE)$_2$-NH$_2$, and Ac-FFKEKEFF-NH$_2$). Each peptide sequence is composed of precisely the same amino acids and is altered only in the primary sequence organization of these residues.

We predicted that each of these peptide sequences would be competent to undergo self-assembly into $\beta$-sheet fibril materials. This prediction was based on the construction of models for predicted $\beta$-sheet strand registry for each peptide (Figure 3.2). Previously reported molecular modeling studies for self-assembled Ac-(FKFE)$_2$-NH$_2$ bilayer nanoribbons indicate that the $\beta$-sheet strand packing mode is antiparallel and out-of-register, leaving the N-terminal Phe residue unpaired at the sheet edges (Figure 3.2A). This out-of-register shift is necessary in order to maintain the segregation of hydrophobic and hydrophilic side chain functionality to opposite faces of the sheet required for bilayer formation. Our models suggest that the Ac-(FK)$_2$(FE)$_2$-NH$_2$ peptide can readily adopt a similar packing structure. These models also indicate that this $\beta$-sheet packing structure for the Ac-(FKFE)$_2$-NH$_2$ and Ac-(FK)$_2$(FE)$_2$-NH$_2$ peptides pairs Lys and Glu side chains cross-strand, providing optimal attractive charge interactions at pH values in which the amine and carboxylic acid groups are ionized.
Figure 3.2. Models for putative antiparallel β-sheet cross-strand registry for Ac-(FKFE)$_2$-NH$_2$ and sequence pattern variants. Phe side chains are shown in green. (A) Antiparallel β-sheet registry for Ac-(FKFE)$_2$-NH$_2$ and Ac-(FK)$_2$(FE)$_2$-NH$_2$ that require a one-residue out-of-register shift in order to maintain exclusively hydrophobic and hydrophilic faces that result in bilayer assembly. This strand registry results in an unpaired N-terminal Phe residue (the so-called “dangling Phe”). (B) Hypothetical in-register antiparallel β-sheet strand registry for the Ac-KEFFFFKE-NH$_2$, Ac-(KFFE)$_2$-NH$_2$, and Ac-FFKEKEFF-NH$_2$ peptides. These β-sheets lack exclusive hydrophobic and hydrophilic faces, but have optimally paired charge groups, aromatic functionality, and hydrogen bond donor/acceptors.

The remaining sequence-altered peptides cannot form β-sheets with exclusively hydrophobic and hydrophilic faces (Figure 3.2B). These changes thus disrupt the potential of these variants to self-assemble into β-sheet bilayer fibrils. Our β-sheet
models for the Ac-KEFFFKFKE-NH₂, Ac-(KFFE)₂-NH₂, and Ac-FFKEKEFF-NH₂ peptides indicate that each peptide is hypothetically able to form in-register antiparallel \( \beta \)-sheets. These \( \beta \)-sheets allow optimal cross-strand pairing of hydrophobic/aromatic Phe side chains as well as complementary charge Lys and Glu residues. These models suggest that self-assembly of each of these sequence-variant peptides can theoretically occur, but they do not allow prediction of the higher order structure for each of the putative \( \beta \)-sheets. While the Ac-(FKFE)₂-NH₂ and Ac-(FK)₂(FE)₂-NH₂ peptides are prone to adopt bilayer structure (\( \beta \)-sheet lamination number of 2), the Ac-KEFFFKFKE-NH₂, Ac-(KFFE)₂-NH₂, and Ac-FFKEKEFF-NH₂ peptides are not obviously subject to the same limited lamination potential. Thus, it was expected that the latter three sequence-variant peptides might self-assemble into fibril structures with distinctive morphologies and emergent properties.

3.3 Results

3.3.1 Peptide self-assembly studies.

All peptides were synthesized as \( N \)-terminal acetyl, \( C \)-terminal amide sequences using standard Fmoc solid phase synthesis techniques (see Experimental Procedures for details and Supporting Information (Figure 3.8.S1–S10, Table 3.8.S1) for characterization data). Purification of peptides via HPLC allowed an opportunity to assess the relative hydrophobicity of each peptide. Under identical mobile and stationary phase conditions, peptide hydrophobicity can be estimated based on HPLC retention times: longer retention times typically correspond to higher hydrophobicity. As expected based on identical amino acid composition (except for sequence order), the peptides were
confirmed to have similar hydrophobicity, with retention times ranging from 11.8–12.4 min (Supporting Information, Figures 3.8.S1–S5).

Peptide self-assembly for all peptides was characterized under several conditions. Self-assembly was assessed at two concentrations, 1.0 mM and 0.2 mM and under two distinct solvent conditions. Peptides were initially dissolved in water lacking exogenous buffer or other salts. Under these conditions the pH ranged from 3–4 due to the presence of residual acid from HPLC purification. At pH 3–4 the peptides are cationic, with a charge of +2. Peptide self-assembly was also studied at neutral pH (30 mM Tris-HCl, pH 7). At neutral pH the peptides are neutral since Glu and Lys side chains are both in an ionized state. Peptides were disaggregated prior to self-assembly analysis by dissolution in acetonitrile/water (60/40, v/v). Peptide concentrations were determined in these acetonitrile/water solutions by HPLC analysis and correlation of integrated peak area to standard concentration curves (see Experimental Procedures for details). Aliquots of the desired peptide quantity were then frozen and lyophilized. Lyophilized peptides were used immediately. Self-assembly was initiated by dissolution into aqueous media and self-assembly was characterized using circular dichroism (CD) and Fourier transform infrared (FT-IR) spectroscopy correlated with transmission electron microscopy (TEM) imaging and electron diffraction.

3.3.2 Spectroscopic analysis of peptide self-assembly.

CD and FT-IR spectroscopy are typically used to assess peptide self-assembly and provide diagnostic spectra of peptide secondary structure. In particular, evolution of $\beta$-sheet secondary structure often corresponds to self-assembly of amphipathic peptides. CD spectra were recorded immediately upon dissolution of peptides in aqueous solutions.
FT-IR spectroscopy in the amide I region is sensitive to water and TFA counterion salts that are present after HPLC purification of synthetic peptides.\textsuperscript{28} Therefore, counterion exchange with HCl must be performed prior to FT-IR analysis. FT-IR must also be recorded in D\textsubscript{2}O in order to eliminate overlap of amide I signals with water. Used in parallel, CD and FT-IR spectroscopy provide reliable assessment of the evolution of $\beta$-sheet secondary structure that is required for self-assembly of amphipathic peptides into amyloid-like fibrils.

The CD spectra for the self-assembled Ac-\((FKFE)\textsubscript{2}-NH\textsubscript{2}\) peptide in water (pH 3–4) were consistent with previously reported data. A maximum at 195 nm and a minimum at 218 nm are indicative of $\beta$-sheet formation (Figure 3.3A).\textsuperscript{28,29,40–42} However, an additional minimum 205 nm is also observed. While this is not indicative of classical $\beta$-sheet structures, this feature of Ac-\((FKFE)\textsubscript{2}-NH\textsubscript{2}\) self-assembled peptides has been attributed to $\pi$-$\pi$ stacking in the hydrophobic core of the putative $\beta$-sheet bilayers\textsuperscript{43} or to slight distortion of the $\beta$-sheets.\textsuperscript{44} CD spectra consistent with self-assembly of the Ac-\((FKFE)\textsubscript{2}-NH\textsubscript{2}\) peptide were observed at both high (1.0 mM) and low (0.2 mM) concentrations.
Figure 3.3. CD spectra for amphipathic peptides at 1.0 mM (red) and 0.2 mM (blue) concentrations in water (pH 3–4). (A) Ac-(FKFE)$_2$-NH$_2$; (B) Ac-(FK)$_2$(FE)$_2$-NH$_2$; (C) Ac-KEFFFFKE-NH$_2$; (D) Ac-(KFE)$_2$-NH$_2$; (E) Ac-FFKEKEFF-NH$_2$. 


The Ac-(FK)$_2$(FE)$_2$-NH$_2$ peptide in water (pH 3–4) exhibited a β-sheet CD-spectrum similar to that of the Ac-(FKFE)$_2$-NH$_2$ peptide. The characteristic peaks for β-sheet formation were observed with a maximum at 195 nm and a minimum at 218 nm (Figure 3.3B). A prominent minimum at 200 nm was also observed, consistent with aromatic or distorted β-sheet effects in the self-assembled materials. A shift of this minimum to 200 nm (from 205 nm for the Ac-(FKFE)$_2$-NH$_2$ peptide) may indicate subtle difference in β-sheet structure between the Ac-(FK)$_2$(FE)$_2$-NH$_2$ and the Ac-(FKFE)$_2$-NH$_2$ self-assembled materials. As with the Ac-(FKFE)$_2$-NH$_2$ peptide, the CD spectra for the Ac-(FK)$_2$(FE)$_2$-NH$_2$ peptide are consistent with β-sheet self-assembly at both high (1.0 mM) and low (0.2 mM) concentrations. These findings indicate that subtle sequence realignment of charged groups in the hydrophilic face does not significantly perturb self-assembly propensity of amphipathic peptides that otherwise maintain a hydrophobic/hydrophilic sequence pattern.

The CD spectra for the remaining sequence-pattern variants in water (pH 3–4) are distinctive. The Ac-KEFFFFKE-NH$_2$ variant had CD signatures that were concentration dependent. At low concentration (0.2 mM), the CD signature of the Ac-KEFFFFKE-NH$_2$ peptide displayed a minimum at 198 nm (Figure 3.3C). This may be consistent with an disordered conformation. At high concentration (1.0 mM), the CD signal changed dramatically, featuring a maximum at 205 nm and broad minimum at 230 nm. This CD signature is not typical for β-sheet, α-helix, or unstructured peptides, implying that unique interactions, such as aromatic π-π effects, dominate the CD signal. As described below, FT-IR studies were necessary to clarify the interpretation of the Ac-KEFFFFKE-NH$_2$ CD spectrum at 1.0 mM concentrations. These CD spectra imply that self-assembly
of the Ac-KEFFFFKE-NH₂ peptide may occur, although only at higher concentrations relative to amphipathic peptides with alternating hydrophobic/hydrophilic sequence patterns.

Lastly, CD spectra of the Ac-(KFFE)₂-NH₂ and Ac-FFKEKEFF-NH₂ sequence variants also lack standard β-sheet signatures in water at pH 3–4. CD spectra of the Ac-(KFFE)₂-NH₂ peptide had a minimum from 205–210 nm with a strong maximum at 195 nm and a weak maximum at 225 nm for both high and low concentrations (Figure 3.3D). This signature is not classically indicative of β-sheet or unstructured peptides; thus, these CD spectra imply that the Ac-(KFFE)₂-NH₂ peptide may be assembled, but clarification via FT-IR spectroscopy and TEM imaging is necessary in order to interpret the CD data. The CD spectra of the Ac-FFKEKEFF-NH₂ peptide (Figure 3.3E) primarily display a strong minimum from 195–200 nm at both high and low concentrations. These spectra may be consistent with disordered, and thus unassembled, states, but clarification by FT-IR and TEM imaging is necessary.

FT-IR in the amide I region is often used in parallel with CD to confirm secondary structure of self-assembled peptides. This is great importance for peptides in which CD spectra are dominated by other electronic effects (for example aromatic interactions) that give signals that are not strictly representative of classical secondary structure signatures. This is the case with the peptides studies herein. FT-IR spectroscopy can also give insight into strand registry in self-assembled peptides, allowing differentiation between antiparallel and parallel orientations in β-sheets.³⁴,³⁹ Accordingly, we performed FT-IR measurements on each peptide studied herein (water, pH 3–4, 1.0 mM). The Ac-(FKFE)₂-NH₂, Ac-(FK)₂(Fe)₂-NH₂, and Ac-KEFFFFKE-NH₂ peptides all displayed a signal at
~1624 cm$^{-1}$ with a weak band from 1680–1690 cm$^{-1}$ (Figure 3.4A). These spectra are clearly consistent with antiparallel $\beta$-sheet structure for all three peptides, indicating that the nonstandard features in the CD spectra for these peptides shown in Figure 3.3C arise from other electronic effects ($\pi-\pi$ interactions or $\beta$-sheet distortion) of the assembled materials.$^{28,45,46}$ Conversely, the Ac-(KFE)$_2$-NH$_2$ and Ac-FFKEKEFF-NH$_2$ variants had broad FT-IR bands at 1645 cm$^{-1}$, consistent with disordered peptides (Figure 3.4B).$^{28,39,47}$
Figure 3.4. FT-IR spectra for sequence-pattern variant peptides at 1.0 mM concentration (water, pH 3–4). (A) β-Sheet structure for the Ac-(FKFE)$_2$-NH$_2$, Ac-(FK)$_2$(FE)$_2$-NH$_2$, Ac-KEFFFFKE-NH$_2$ peptides. (B) Disordered structure for the Ac-(KFE)$_2$-NH$_2$ and Ac-FFKEKEFF-NH$_2$ peptides.

CD and FT-IR data suggest that sequence pattern exerts a profound effect on the self-assembly of amphipathic peptides. CD and FT-IR data concur that amphipathic peptides with repeating hydrophobic/hydrophilic sequence patterns, Ac-(FKFE)$_2$-NH$_2$ and Ac-(FK)$_2$(FE)$_2$-NH$_2$, readily adopt β-sheet secondary structures across a range of peptide
concentrations. The Ac-KEFFFFKE-NH$_2$ peptide, which organizes the Phe residues into a hydrophobic core in the center of the putative $\beta$-strand, can also adopt $\beta$-sheet structures, although only at high concentrations. Lastly, the Ac-KFFFKFFFE-NH$_2$ and Ac-FFKEKEFF-NH$_2$ fail to assume $\beta$-sheet character, presumably indicating that these sequence patterns have a lower propensity to self-assemble. Additional imaging studies were conducted in order to enable interpretation of these suggestive spectroscopic analyses.

### 3.3.3 TEM imaging of self-assembled structures.

The use of microscopic imaging techniques is required to confirm spectroscopic evidence for self-assembly and to characterize the morphology of the resulting self-assembled materials. Accordingly, we obtained transmission electron microscopy (TEM) images for solutions of each peptide (water, pH 3–4) at both high and low concentrations (1.0 mM and 0.2 mM, respectively) (Figure 3.5). The Ac-(FKFE)$_2$-NH$_2$, peptide was found to self-assemble into helical nanoribbon structures at both high and low concentrations (Figure 3.5A-B), as has been previously reported.$^{21,22,25,28,48}$ These helical nanoribbons were 8.7 ± 0.6 nm and 8.5 ± 0.6 nm wide with a helical pitch of 18.8 ± 1.8 nm 19.6 ± 1.9 nm at 1.0 mM and 0.2 mM concentrations, respectively (Table 3.1). These nanoribbons are micrometers in length. The helical structures relax into flat nanoribbons over time (days).$^{21}$ A slightly different morphology was observed in TEM images of self-assembled Ac-(FK)$_2$(FE)$_2$-NH$_2$ peptides (Figure 3.5C-D). Ac-(FK)$_2$(FE)$_2$-NH$_2$ also self-assembles into micrometers-long, flat nanoribbon structures, but with narrower widths approximately half those of the Ac-(FKFE)$_2$-NH$_2$ nanoribbons (4.2 ± 0.6 nm and 3.8 ± 0.6 nm for 1.0 mM and 0.2 mM, respectively). No obvious helical structure is detected
for these narrower nanoribbons. The slight variation between the CD signatures of Ac-(FKFE)$_2$-NH$_2$ and Ac-(FK)$_2$(FE)$_2$-NH$_2$ nanoribbons must arise from the subtle difference in molecular packing that give rise to these similar, but distinct, morphologies. The molecular basis for these differences in morphology is not immediately apparent and will be the subject of future study.

**Figure 3.5.** Negative stain TEM images for sequence pattern variants (water, pH 3–4). Ac-(FKFE)$_2$-NH$_2$ at 0.2 mM (A) and 1.0 mM (B); Ac-(FK)$_2$(FE)$_2$-NH$_2$ at 0.2 mM (C) and 1.0 mM (D); Ac-KEFFFFKE-NH$_2$ at 0.2 mM (E) and 1.0 mM (F); Ac-(KFFE)$_2$-NH$_2$ at 0.2 mM (G) and 1.0 mM (H); Ac-FFKEKEFF-NH$_2$ at 0.2 mM (I) and 1.0 mM (J).

**Table 3.1.** Fibril morphologies for sequence variant peptides at varying concentration and solvent conditions.

<table>
<thead>
<tr>
<th></th>
<th>Ac-(FKFE)$_2$-NH$_2$</th>
<th>Ac-(FK)$_2$(FE)$_2$-NH$_2$</th>
<th>Ac-KEFFFFKE-NH$_2$</th>
<th>Ac-FFKEKEFF-NH$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dimensions (nm)</td>
<td>Dimensions (nm)</td>
<td>Dimensions (nm)</td>
<td>Dimensions (nm)</td>
</tr>
<tr>
<td>Width (pH 3–4)</td>
<td>8.7 ± 0.6</td>
<td>8.5 ± 0.6</td>
<td>4.2 ± 0.6</td>
<td>22.5 ± 3.7</td>
</tr>
<tr>
<td>Width (pH 7)</td>
<td>8.8 ± 0.9</td>
<td>4.3 ± 0.9</td>
<td>23.6 ± 3.3</td>
<td>25.2 ± 3.1</td>
</tr>
<tr>
<td>Helical pitch</td>
<td>18.8 ± 1.8</td>
<td>19.6 ± 1.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fibril height</td>
<td>-</td>
<td>-</td>
<td>3.6 ± 0.6</td>
<td>-</td>
</tr>
</tbody>
</table>
TEM images clarify the spectroscopic signatures of Ac-KEFFFFKE-NH$_2$, Ac-(KFFE)$_2$-NH$_2$, and Ac-FFKEKEFF-NH$_2$ peptides. At 0.2 mM concentrations, TEM images for all three of these peptides lack evidence of higher order assembled structures (Figure 3.5E, G, I). Thus, based on spectroscopic and microscopic evidence, these changes in sequence pattern have a significant effect on the self-assembly propensity of these materials. Self-assembly must be viewed as less thermodynamically favorable for each of these peptides relative to the hydrophobic/hydrophilic-patterned sequences, since no self-assembly is observed at low concentrations. At high concentrations (1.0 mM), neither Ac-(KFFE)$_2$-NH$_2$ and Ac-FFKEKEFF-NH$_2$ peptides were observed to efficiently form β-sheet fibril/tape-like structures, consistent with CD and FT-IR evidence that these peptides remain random in secondary structure (Figure 3.5H, J). Interestingly, some evidence of micelle-like aggregate formation is observed for these peptides, with micelle structures seen much more prominently in solutions of the Ac-FFKEKEFF-NH$_2$ peptide (Figure 3.5J). This is consistent with CD spectra that suggest some type of higher order structure, albeit not β-sheet, may exist in the Ac-FFKEKEFF-NH$_2$ peptide. Finally, at 1.0 mM concentrations at which CD/FT-IR spectroscopy indicates β-sheet structure for the Ac-KEFFFFKE-NH$_2$ peptide, unique nanotape structures are observed (Figure 3.5F). These broad nanotapes are 22.5 ± 3.6 nm wide with a tape height of 3.6 ± 0.6 nm that can be measured at points where the tapes twist.

Electron diffraction studies were also performed on TEM grids on which higher order aggregates were observed. The resulting electron diffraction patterns (Supporting Information, Figures 3.8.S11) were used to calculate the distance ($d$-spacing) between β-strands (Supporting Information, Figure 3.8.S12). Peptides that self-assemble into β-sheet
structures have a $d$-spacing of ~4.7 Å corresponding to the distance between neighboring $eta$-strands within $eta$-sheets. The hydrophobic/hydrophilic-patterned amphipathic $eta$-sheet peptides, Ac-(FKFE)$_2$-NH$_2$ and Ac-(FK)$_2$(FE)$_2$-NH$_2$, had $d$-spacings of ~4.7 Å (Supporting Information, Figures 3.8.S.12). The Ac-KEFFFFKE-NH$_2$ had prominent scattering corresponding to ~4.7 Å $d$-spacing values, further confirming that the observed broad nanotape structure is composed of $eta$-sheet oriented peptides. Lastly, electron diffraction of the Ac-FFKEKEFF-NH$_2$ micelle-like structures failed to give prominent scattering intensities, suggesting that the micelles are composed of random structured peptides.

### 3.3.4 Self-assembly at neutral pH.

The effects of pH on self-assembly of these sequence pattern variants was assessed by examining these peptides in buffered aqueous solutions (30 mM Tris-HCl, pH 7). A change in pH is often used as an external stimulus to promote self-assembly.$^{50-52}$ The peptides studied herein have a +2 charge at pH 3–4; this charge will be neutralized at pH 7 as a function of Glu deprotonation. At neutral pH it was found that sequences that undergo self-assembly precipitated; this is in contrast to studies at pH 3–4 in which all materials, both monomeric and self-assembled, were water-soluble. Precipitation complicated spectroscopic analyses of these materials due to light scattering and sedimentation of the assembled materials. However, TEM analysis of the suspended precipitate allowed us to qualitatively assess the effects of pH on self-assembly of these materials.

Similar self-assembly behavior was observed at neutral pH as was seen at acidic pH, with a few notable differences. It was found that the Ac-(FKFE)$_2$-NH$_2$ and Ac-
(FK)$_2$(FE)$_2$-NH$_2$ also efficiently self-assemble at neutral pH. However, some moderate changes in fibril morphology were observed as a consequence of self-assembly at pH 7. The helical nanoribbons formed by Ac-(FKFE)$_2$-NH$_2$ at pH 3–4 were less common at neutral pH; flattened nanoribbons of the same dimensions instead dominated these images (dimensions were 8.7 ± 0.6 nm at pH 4 and 8.8 ± 0.9 nm at pH 7 respectively) (Figure 3.6A, Table 3.1). The morphology of Ac-(FK)$_2$(FE)$_2$-NH$_2$ nanoribbons appeared to be unaffected by variations in pH (Figure 3.6B). At neutral pH, a high degree of fibril bundling was observed perhaps due to neutralization of the nanoribbon surface, thus reducing ribbon-ribbon repulsion that may be observed in water at pH 3-4 (positively charged fibrils at low ionic strength are less likely to form close surface interactions with neighboring fibrils). Precipitation of the self-assembled materials at neutral pH is probably due to a combination of higher order nanoribbon bundling and also due to the reduced surface charge (and thus lower inherent solubility) of the assembled fibrils.
Figure 3.6. Negative stain TEM images for self-assembled peptides at pH 7 (30 mM Tris-HCl, 1.0 mM peptide). (A) Ac-(FKFE)2-NH2; (B) Ac-(FK)2(FE)2-NH2; (C) Ac-KEFFFFKE-NH2; (D) Ac-FFKEKEFF-NH2.

Self-assembly for amphipathic sequence pattern-variants was more dramatically affected at neutral pH. At neutral pH, the Ac-KEFFFFKE-NH2 peptide self-assembled into ~20 nm wide nanotapes similar to those observed at acidic pH (Figure 3.6C). However, these nanotapes were found to be highly bundled with neighboring tapes, resulting in a more rigid material that lacked the gentle twisting morphology of the nanotapes observed at acidic pH. The Ac-(KFFE)2-NH2 also failed to form higher order structures at neutral pH at concentrations up to 1.0 mM (images not shown). However, increasing the pH promoted efficient self-assembly of the Ac-FFKEKEFF-NH2 peptide into ~20 nm wide nanoribbons (Figure 3.6D) similar to those observed for the Ac-KEFFFFKE-NH2 peptide. Thus, an increase in pH facilitated a transition from micelle-
like aggregates (pH 4) to nanotapes (pH 7). Electron diffraction analysis of all assembled peptides indicated 4.7 Å \( d \)-spacing values (Supporting Information, Figure 3.8.S13-S14) consistent with \( \beta \)-sheet structures. Thus, self-assembly of sequence-pattern variants of amphipathic peptides is sensitive, to some extent, to environment.

3.3.5 Sedimentation analysis to assess thermodynamic effects on self-assembly.

Peptide self-assembly is an equilibrium process. The final equilibrium state can be expressed as fibril\(_n\) + [monomer] \( \rightleftharpoons \) fibril\(_{n+1}\).\(^{53-57}\) The concentration of monomer at equilibrium is defined as the critical concentration \((C_r)\); \(C_r\) is an inherent property of any self-assembling peptide as a function of self-assembly conditions. Thus, under any given conditions of solvent, ionic strength, temperature, etc., the \(C_r\) defines the minimum concentration necessary to promote self-assembly of the given peptide.\(^{56-58}\) That is, self-assembly does not occur at concentrations below the \(C_r\) for any peptide under the conditions that \(C_r\) is measured. Measurement of \(C_r\) has been used to assess the thermodynamics of amyloid self-assembly.\(^{56-58}\) When comparing amyloid peptides of similar sequence (for example single amino acid mutants) that self-assemble into fibrils that have the same \( \beta \)-sheet packing orientation, \(C_r\) can be used to derive equilibrium association constants \((K_a)\) for addition of one molecule of monomer to a fibril end. By extension, these \(K_a\) values can be used to infer comparative \(\Delta G\) values in order to assess the thermodynamic contributions of a given amino acid to self-assembly into amyloid or amyloid-like fibrils.

In order to understand the effects that sequence patterning has on the thermodynamics of amphipathic peptide self-assembly, we have measured the equilibrium \(C_r\) values for each sequence pattern variant peptide studied herein. TEM images show dramatic
morphological differences between the materials assembled from the various peptides herein, implying differences in overall packing architecture. Thus, the meaning of any calculated thermodynamic parameters ($K_a$, $\Delta G$) derived from measured $C_r$ values for these materials are difficult to interpret. As such, we simply report the measured $C_r$ values. In general, it can be assumed that a lower $C_r$ value corresponds to a more favorable self-assembly propensity for a given peptide under the comparison conditions.

Equilibrium $C_r$ values were measured by adapting Wetzel’s HPLC sedimentation assay.\textsuperscript{59} Self-assembly for each peptide was initiated by addition of water to the lyophilized peptides at starting concentrations of either 0.2 mM or 1.0 mM and the resulting solutions were allowed to equilibrate over 24 h (assembly pH ranges from 3–4). Controls for each peptide at both concentrations were prepared by dissolving peptides in acetonitrile/water with 0.1% TFA (60% acetonitrile by volume); under these conditions the peptides do not self-assemble. Aliquots removed from each solution were subjected to centrifugation to sediment any aggregated material and a sample of the supernatant was carefully removed. The concentration of monomer in the supernatant ($C_r$) was determined by injection of the aliquot onto an analytical HPLC instrument and correlation of the integrated peak area to a standard curve (see Experimental Procedures for details).

The measured equilibrium $C_r$ values are compiled in Table 3.2. The parent peptide, Ac-(FKFE)$_2$-NH$_2$, had the lowest $C_r$ values of ~50 µM ($49 \pm 4$ µM at 0.2 mM starting peptide concentration and $54 \pm 10$ µM at 1.0 mM starting peptide concentration). As expected, these values are nearly identical since $C_r$ is only dependent on assembly conditions. It was expected that the Ac-(FK)$_3$(FE)$_2$-NH$_2$ variant would have similar $C_r$ values to the parent peptide. However, despite the similar amphipathic $\beta$-sheet sequence
patterns, the slight change in amino acid composition on the hydrophilic face caused almost a three-fold increase in $C_r$ for the Ac-(FK)$_2$(FE)$_2$-NH$_2$ variant: 138 ± 25 µM (0.2 mM) and 145 ± 17 µM (1.0 mM). Studies involving the (AEAEAKAK)$_2$ peptide and a derivative with an identical amino acid composition, (AE)$_4$(AK)$_4$, also showed variations in $C_r$ by approximately three-fold. The reason for these discrepancies are not completely understood and require further studies to determine the factors leading to the decreased self-assembling propensity for Ac-(FK)$_2$(FE)$_2$-NH$_2$. It can be hypothesized, however, that the altered nanoribbon morphology for the Ac-(FK)$_2$(FE)$_2$-NH$_2$ variant results in β-sheet packing that is, in some way, thermodynamically destabilized relative to the Ac-(FKFE)$_2$-NH$_2$ nanofibrils. The thinner Ac-(FK)$_2$(FE)$_2$-NH$_2$ β-sheet nanoribbons are approximately a single peptide in width, whereas the wider Ac-(FKFE)$_2$-NH$_2$ nanoribbons must feature several β-sheets oriented side-by-side running parallel to the nanoribbon axis. This more extensive side-by-side arrangement represents higher order assembly events that must impart a thermodynamic advantage to these materials.

### Table 3.2. Measured $C_r$ values for amphipathic sequence pattern variants.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$C_r$ (µM) (0.2 mM)</th>
<th>$C_r$ (µM) (1.0 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-(FKFE)$_2$-NH$_2$</td>
<td>49 ± 4</td>
<td>54 ± 10</td>
</tr>
<tr>
<td>Ac-(FK)$_2$(FE)$_2$-NH$_2$</td>
<td>138 ± 25</td>
<td>145 ± 17</td>
</tr>
<tr>
<td>Ac-KEFFFFKE-NH$_2$</td>
<td>174 ± 26</td>
<td>517 ± 61</td>
</tr>
<tr>
<td>Ac-(KFFE)$_2$-NH$_2$</td>
<td>185 ± 16</td>
<td>1004 ± 40</td>
</tr>
<tr>
<td>Ac-FFKEKEFF-NH$_2$</td>
<td>200 ± 4</td>
<td>864 ± 32</td>
</tr>
</tbody>
</table>
Spectroscopic and imaging analysis of Ac-KEFFFKKE-NH₂ self-assembly indicated self-assembly occurs at 1.0 mM but not at 0.2 mM starting concentrations in water at pH 3–4. Consistent with these observations is was found that Ac-KEFFFKKE-NH₂ had measured $C_r$ values of $174 \pm 26$ µM (0.2 mM peptide) and $517 \pm 61$ µM (1.0 mM peptide). Thus, at 0.2 mM peptide, virtually all the material is in a soluble, unassembled state since the critical concentration to promote self-assembly is ~0.5 mM. These results indicate that this peptide has a significantly reduced propensity to self-assemble relative to sequences with alternating hydrophobic/hydrophilic sequence patterning.

As expected, the Ac-(KFFE)$_2$-NH₂ and Ac-FFKEKEFF-NH₂ peptides had equilibrium $C_r$ values that were near or above 1.0 mM under these solvent conditions (water, pH 3–4). The Ac-(KFFE)$_2$-NH₂ peptide that remained unassembled at both high and low peptide concentrations had $C_r$ values that confirmed the spectroscopic and TEM analysis ($185 \pm 16$ µM (0.2 mM) and $1004 \pm 40$ µM (1.0 mM)). Lastly, the Ac-FFKEKEFF-NH₂ peptide had measured $C_r$ values of $200 \pm 4$ µM (0.2 mM) and $864 \pm 32$ µM (1.0 mM). There was TEM evidence that Ac-FFKEKEFF-NH₂ formed micelle-like aggregates at 1.0 mM. $C_r$ measurements suggest that the critical micelle concentration for the Ac-FFKEKEFF-NH₂ peptide is near the 1.0 mM starting concentration used in these experiments. These measurements confirm that sequence patterns that feature Phe-Phe dipeptide motifs separated by hydrophilic amino acid linkers have severely impeded self-assembly properties relative to the hydrophobic/hydrophilic-repeating pattern.

### 3.4 Discussion

The results reported herein indicate that sequence pattern has a dramatic effect on amphipathic peptide self-assembly into β-sheet materials. Five amphipathic peptides
were analyzed that were composed of identical amino acids, varying only in the arrangement of sequence pattern. These peptides included two that retained the hydrophobic/hydrophilic repeating pattern that has been repeatedly shown to promote β-sheet self-assembly (Ac-(FKFE)\_2-NH\_2 and Ac-(FK\_2(FE)\_2-NH\_2). The remaining three variants (Ac-KEFFFFKE-NH\_2, Ac-(KFFE)\_2-NH\_2, and Ac-FFKEKEFF-NH\_2) were designed to preserve complementary electrostatic interactions and π-π stacking in hypothetical antiparallel β-sheet packing modes and feature Phe-Phe dipeptides separated by 0, 1, or 2 hydrophilic Glu-Lys pairs. It was found that peptides with alternating hydrophobic/hydrophilic patterning underwent the most efficient self-assembly. Both Ac-(FKFE)\_2-NH\_2 and Ac-(FK\_2(FE)\_2-NH\_2 formed β-sheet nanoribbons at both low and high concentrations. The remaining sequence patterns either failed to self-assemble altogether (Ac-(KFFE)\_2-NH\_2) or self-assembled into broad, morphologically distinct β-sheet nanotapes at high concentrations (Ac-KEFFFFKE-NH\_2) or under higher pH conditions (Ac-FFKEKEFF-NH\_2).

There may be several reasons for the severely reduced self-assembly propensity of peptides that lack strict hydrophobic/hydrophilic sequence patterns. The inherent β-sheet propensity of the resulting sequences may offer a primary explanation for these results. Phe has high β-sheet propensity whereas Glu and Lys have relatively low β-sheet propensity (Glu and Lys are statistically more likely to be found in α-helical structures).\textsuperscript{63,64} Peptides in which the Phe and Glu/Lys residues are strictly alternating have high β-sheet propensity in part due to the evenly spaced arrangement of β-sheet-prone Phe residues. This arrangement balances the low β-sheet potential of the hydrophilic amino acids. For peptides in which Phe is clustered, the overall inherent
sequence β-sheet propensity is altered. The Ac-KEFFFKKE-NH$_2$ peptide, which self-assembled into nanotapes at higher concentrations (1 mM) may have an advantage among the sequence-pattern variants since it offers a core of four β-sheet-prone Phe residues flanked by solubilizing hydrophilic groups with lower sheet potential. This central Phe core may effectively facilitate adoption of β-sheet structures. In comparison, peptides in which Phe-Phe motifs are interrupted by Glu-Lys motifs (Ac-(KFFE)$_2$-NH$_2$, and Ac-FFKEKEFF-NH$_2$) may suffer from lower inherent sheet potential, thus reducing the overall self-assembly propensity.

A second advantage of hydrophobic/hydrophilic sequence patterned amphipathic peptides in regards to self-assembly potential lies in the structure of the resulting β-sheets. Peptides such as Ac-(FKFE)$_2$-NH$_2$ and Ac-(FK)$_2$(FE)$_2$-NH$_2$ adopt β-sheets that segregate all hydrophobic functionality to one face of the resulting sheet and all hydrophilic functionality to the opposite face. Formation of β-sheet bilayer structures as depicted in Figure 3.1 allows efficient burial of hydrophobic amino acids in the hydrophobic interior of the bilayer. As shown by the reported sedimentation analysis, this provides a significant thermodynamic driving force in favor of self-assembly. In addition, the presentation of hydrophobic groups on a single face of these sheets likely hastens hydrophobic collapse into assembled structures, providing kinetic advantages to self-assembly in addition to thermodynamic advantages.

The altered sequence patterns of the Ac-KEFFFKKE-NH$_2$, Ac-(KFFE)$_2$-NH$_2$, and Ac-FFKEKEFF-NH$_2$ patterns lack the advantage of segregating hydrophobic groups to a single face of hypothetical β-sheets (Figure 3.2). Thus, the structural possibility of forming bilayer structures in which all hydrophobic groups are buried in a continuous
hydrophobic core is not available for these peptides. Having hydrophilic neighboring groups on the same face as hydrophobic Phe side chains in the β-strand structured peptides may reasonably be expected to reduce the driving force for desolvation and hydrophobic collapse for these smaller hydrophobic patches. In this regard, it seems significant that of these pattern-variant peptides, the Ac-KEFFFFKE-NH₂, which possesses a continuous stretch of Phe residues, is the most prone to self-assembly. Interestingly, the other sequence that can be induced to self-assemble under higher pH conditions, the Ac-FFKEKEFF-NH₂ peptide, also has Phe residues that are at the peptide edges without neighboring hydrophilic groups.

The Phe-Phe dipeptide and peptides containing Phe-Phe motifs have been shown to efficiently undergo self-assembly. For example, while the Ac-(KFFE)₂-NH₂ did not assemble under the conditions studied herein, similar peptides have been reported that do assemble. The KFFE tetrapeptide⁶⁵ and the KFEAAAKKFFÉ peptide³¹ have both been shown to self-assemble into β-sheet fibril materials, although self-assembly of these peptides occurred at higher concentrations, under different conditions, and over extended time periods compared to those reported herein. Based on these precedents, the Ac-(KFFE)₂-NH₂ peptide may be capable of self-assembly although under different conditions that those reported herein. Similarly, peptides with terminus-flanking Phe-Phe motifs such as the FFKLVFF peptide,⁶⁶ undergo self-assembly under the proper conditions. As illustrated in this work, the Ac-FFKEKEFF-NH₂ peptide can effectively self-assemble, but only at neutral pH. Thus, it should be stressed that the results reported herein are not intended to comment on the universal ability of the reported to self-
assemble, but rather are meant to be comparative assessments of self-assembly propensity under a strictly defined set of conditions.

Another interesting consequence of sequence pattern variation is the corresponding variation in the morphology of the assembled materials. Varying fibril morphology presumably indicates molecular level variation of peptide packing within the β-sheet structures. The differences between Ac-(FKFE)$_2$-NH$_2$ and Ac-(FK)$_2$(FE)$_2$-NH$_2$ nanoribbons are worthy of additional discussion (the former are 8 nm in width, while the latter are 4 nm in width). The reasons for these morphological differences are not readily apparent when considering possible cross-strand orientations within the respective nanoribbons. As depicted in the β-sheet models shown in Figure 3.2, these two peptides should adopt similar packing modes. In previous investigations of ionic complementary amphipathic β-sheet peptides, Zhang et al. and Saiani et al. have reported that (FE)$_2$(FK)$_2$, (FEFK)$_2$ and related peptides formed identical fibril types, albeit under different solvent conditions and at higher concentrations (~3–8 mg ml$^{-1}$) than were utilized herein.$^{45,67}$ The nanoribbon widths of Ac-(FK)$_2$(FE)$_2$-NH$_2$ are approximately half the width of Ac-(FKFE)$_2$-NH$_2$. The 4 nm wide Ac-(FK)$_2$(FE)$_2$-NH$_2$ ribbons are approximately the same width as the peptide length, implying that these nanoribbons are composed of β-sheets that are precisely one peptide wide. In comparison, the 8 nm Ac-(FKFE)$_2$-NH$_2$ nanoribbons must be composed of two β-sheets aligned edge-to-edge (Figure 3.7). It is uncertain why these subtle changes in sequence would give rise to these morphological effects. It is possible that the block effect of grouped charges in the Ac-(FK)$_2$(FE)$_2$-NH$_2$ peptide results in tighter sheet packing with less “slip” in register, thus limiting the sheet packing to a single sheet in width. This raises the question of why this
would then result in higher critical concentrations for self-assembly of Ac-(FK)$_2$(FE)$_2$-NH$_2$ compared to Ac-(FKFE)$_2$-NH$_2$. These problems are the subjects of future studies.

![Figure 3.7: Lamination modes that give rise to nanoribbon/nanotape morphologies: edge-to-edge vs. face-to-face association of β-sheet structures.](image)

**Figure 3.7.** Lamination modes that give rise to nanoribbon/nanotape morphologies: edge-to-edge vs. face-to-face association of β-sheet structures.

Altering the hydrophobic/hydrophilic faces of amphipathic β-strand peptides by varying the sequence pattern of amino acids gives rise to other possibilities for β-sheet self-assembly. Self-assembly of the Ac-KEFFFFKE-NH$_2$ and Ac-FFKEKEFF-NH$_2$ peptides results in the formation of ~20 nm nanotapes that are from 3–4 nm in height. There are several possible packing modes that can account for these interesting morphologies. First, these peptides may form β-sheet bilayers that associate edge-to-edge
(5 β-sheets) to provide the observed tape structures (Figure 3.7). However, because these peptides cannot form β-sheets with exclusive hydrophobic and hydrophilic faces, self-assembly is not limited to bilayer structures. It is therefore possible that sheet lamination occurs face-to-face (Figure 3.7) to give rise to the observed nanotapes. Amyloid peptides are known to have sheets that laminate face-to-face in this manner. Thus, it is significant that a consequence of varying the sequence pattern of amphipathic peptides is variation in higher order sheet assembly, which can give rise to radically altered fibril morphologies. Precise molecular descriptions for varying morphologies of the self-assembled materials reported herein will require additional structural studies.

3.5 Conclusion

Herein we have reported an analysis of the effect of amino acid sequence pattern on the self-assembly of amphipathic peptides into β-sheet materials. These studies confirm that the optimal amino acid sequence pattern for promotion of β-sheet self-assembly of amphipathic peptides is composed of alternating hydrophobic (X) and hydrophilic (Z) amino acids in a general (XZXZ)ₙ motif. Varying amino acid groupings provided peptides that, despite having identical amino acid constituents and overall hydrophobicity, exhibited severely reduced self-assembly propensity. Alternative sequence patterns (Ac-KEFFFKKE-NH₂ and Ac-FFKEKEFF-NH₂) were able to form β-sheet materials, but only at much higher peptide concentrations relative to (XZXZ)ₙ-patterned peptides. Significantly, the Ac-KEFFFKKE-NH₂ and Ac-FFKEKEFF-NH₂-derived β-sheet materials were of unique broad nanotape morphology relative to the thinner nanoribbons formed by (XZXZ)ₙ-patterned peptides. These results indicate that
amino acid sequence patterns exert a profound influence on self-assembly propensity and morphology of the resulting materials even when the overall hydrophobicity or charge of the related peptides are identical. Sequence pattern variation can thus be exploited as a variable in the creation of novel materials composed of self-assembled peptides. Future work will focus on understanding the molecular basis for these observations.

3.6 Experimental Procedures

3.6.1 Peptide synthesis, purification, and characterization.

Peptides were synthesized manually or on a microwave-equipped Liberty Peptide Synthesizer (CEM®) using standard Fmoc chemistry with HBTU/HOBt activation. Rink amide resin (Advanced ChemTech, 100-200 mesh, 0.2 mmol g⁻¹) was used as the solid support to provide C-terminal amide peptides. Peptides were capped with 20% acetic anhydride in DMF to give N-terminal acetylation. Peptides were cleaved manually from the solid support using trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water (95:2.5:2.5, v/v/v) at room temperature for one hour followed by evaporation of ~90% of the cleavage cocktail. Peptides were precipitated in cold ether and collected by centrifugation. Peptides were then dissolved in DMSO for HPLC purification.

HPLC purification of peptides was conducted using a Shimadzu LC-AD HPLC instrument over a reverse phase C18 column (Waters, BEH300, 10 µm, 19 × 250 mm). Peptides were eluted with a binary gradient of 10 mL min⁻¹ in acetonitrile/water with 0.1% TFA and eluent was monitored by UV absorbance at 215 nm and 254 nm. Fractions were collected and lyophilized then injected on an analytical HPLC with a reverse phase C18 column (Waters, BEH300, 10 µm, 4.6 × 250 mm) to confirm purity (Figure 3.8.S1-S). Peptide identity was confirmed by MALDI-TOF mass spectroscopy (Figure 3.8.S6-
10 and Table 3.8.S1). Ac-(FKFE)$_2$-NH$_2$ $m/z$ 1162.78 (1162.34 calcd for [MH]$^+$), $m/z$ 1184.25 (1184.34 calcd for [MNa]$^+$), $m/z$ 1200.20 (1200.43 calcd for [MK]$^+$). Ac-(FK)$_2$(FE)$_2$-NH$_2$ $m/z$ 1162.77 (1162.34 calcd for [MH]$^+$), $m/z$ 1184.77 (1184.34 calcd for [MNa]$^+$), $m/z$ 1200.75 (1200.43 calcd for [MK]$^+$). Ac-KEFFFKE-NH$_2$ $m/z$ 1162.74 (1162.34 calcd for [MH]$^+$), $m/z$ 1184.73 (1184.34 calcd for [MNa]$^+$), $m/z$ 1200.70 (1200.43 calcd for [MK]$^+$). Ac-(KFFE)$_2$-NH$_2$ $m/z$ 1162.72 (1162.34 calcd for [MH]$^+$), $m/z$ 1184.71 (1184.34 calcd for [MNa]$^+$), $m/z$ 1200.67 (1200.43 calcd for [MK]$^+$). Ac-FFKEKEFF-NH$_2$ $m/z$ 1162.77 (1162.34 calcd for [MH]$^+$), $m/z$ 1184.76 (1184.34 calcd for [MNa]$^+$), $m/z$ 1200.74 (1200.43 calcd for [MK]$^+$).

3.6.2 Peptide self-assembly.

Peptide self-assembly was analyzed in water (pH 3–4) and in 30 mM Tris-HCl solution (pH 7). Self-assembly was characterized at peptide concentrations of 1.0 mM and 0.2 mM. Peptides were dissolved in acetonitrile/water and peptide concentration was determined by correlation of HPLC peak area to a standard curve constructed for each peptide.$^{28}$ Absolute concentration of standard curves was determined by amino acid analysis (AIBiotech, Richmond, VA). Once concentration was determined, peptides were distributed in required quantities and these samples were frozen and lyophilized. Self-assembly was initiated by dissolving lyophilized peptides in water (1.0 mM and 0.2 mM concentrations) or in Tris-HCl solution (pH 7). Upon addition of solvent, peptides were subjected to three cycles of vortex (1 min) and sonication (5 min). Self-assembly was monitored by circular dichroism (CD) and Fourier transform infrared (FT-IR) spectroscopy correlated to transmission electron micrograph (TEM) images as described below.
3.6.3 Circular dichroism (CD) spectroscopy.

CD spectra were recorded on an AVIV 202 circular dichroism spectrometer ~20 min after dissolution of the peptides in water. Spectra were obtained using a 37 µL aliquot of peptide on a 0.1 mm path length quartz cuvette (Hellma) at 25 °C from 260 to 190 nm with 1.0 nm step, 1.0 nm bandwidth, and a 3 second collection time per step. The background subtraction, molar ellipticity conversion, and data smoothing with a least squares fit were processed using the AVIV software.

3.6.4 Fourier transform infrared (FT-IR) spectroscopy.

FT-IR spectra were obtained ~20 min after peptide dissolution using a Shimadzu 8400 FT-IR spectrometer. Residual TFA was removed by anion exchange by lyophilization from acetonitrile/water/HCl (4:5.9:0.1, v/v/v). Peptides were then lyophilized twice from deuterium oxide to remove residual water. Lyophilized peptides were dissolved in D₂O (pH 4 ± 0.5). An aliquot (70 µL) of each peptide was placed on 25 mm × 4 mm CaF₂ plates (International Crystal Labs). Background and absorbance measurements were taken using the Happ-Genzel method from 1550–1750 cm⁻¹ with a 4 cm⁻¹ resolution for 1024 scans.

3.6.5 Negative-stain transmission electron microscopy (TEM) and electron diffraction (ED).

An aliquot (10 µL) of assembled peptide was applied to a 200-mesh carbon-coated copper grid for 1 min. The excess fluid was removed by capillary action. For 1.0 mM samples, the assembled peptide was diluted to 0.20 mM prior to staining in order to reduce fibril concentration on the grid. Samples prepared in Tris-HCl buffer were washed with water (10 µL) which was removed by capillary action to remove salt before staining. The grids were stained by applying 5% uranyl acetate (10 µL) for 1 min followed by
removal by capillary action. The stained grids were air-dried prior to imaging. A Hitachi 7650 transmission electron microscope in high-contrast mode was used at an accelerating voltage of 80 kV to obtain electron micrographs. Electron diffraction experiments were performed on the TEM grids at a spot size of 30 nm and a camera length of 2 m. Fibril dimensions were determined by performing at least 100 measurements on unique fibrils and diffraction profiles to define the \( d \)-spacings for each peptide was calculated using the program ImageJ from the NIH website. (http://rsbweb.nih.gov/ij/).

3.6.6 Sedimentation analyses.

Sedimentation analysis was used to understand the equilibrium between monomer and fibril for each peptide. This was accomplished by modifying Wetzel’s HPLC sedimentation assay that has been used to study the thermodynamic effects of substitutions on amyloid self-assembly. Peptides were incubated at 1.0 mM and 0.2 mM in water (pH 3–4) for 24 h at room temperature to allow them to come to equilibrium. Unassembled control experiments were prepared at identical concentrations in 60% acetonitrile/water (by volume) with 0.1% TFA (these solutions prevent self-assembly of amphipathic peptides). Peptide solutions and control solutions were subjected to centrifugation (100,000 g) for 1 h at 4 °C to sediment aggregate materials. An aliquot of the supernatant of the centrifuged peptide solution was carefully removed from the upper one-third volume of the centrifuged material; this aliquot was diluted 20 fold in DMSO then injected in triplicate on a Shimadzu analytical HPLC (reverse phase C18 column, Waters, BEH300, 10 mm, 4.6 × 250 mm). The unassembled monomer concentration was determined by correlation of integrated peak area to an HPLC standard concentration curve.28,59
3.7 References


3.8 APPENDIX: Supporting Information and Data

Figure 3.8.S1. Analytical HPLC trace for Ac-(FKFE)_2-NH₂.

![Figure 3.8.S1. Analytical HPLC trace for Ac-(FKFE)_2-NH₂.](image)

Figure 3.8.S2. Analytical HPLC trace for Ac-(FK)_2(Fe)_2-NH₂.

![Figure 3.8.S2. Analytical HPLC trace for Ac-(FK)_2(Fe)_2-NH₂.](image)

Figure 3.8.S3. Analytical HPLC trace for Ac-KEFFFKFKE-NH₂.

![Figure 3.8.S3. Analytical HPLC trace for Ac-KEFFFKFKE-NH₂.](image)
Figure 3.8.S4. Analytical HPLC trace for Ac-(KFFE)$_2$-NH$_2$.

Figure 3.8.S5. Analytical HPLC trace for Ac-FFKEKEF-NH$_2$.

Figure 3.8.S6. MALDI-TOF-MS data for Ac-(FKFE)$_2$-NH$_2$.
Figure 3.8.S7. MALDI-TOF-MS data for Ac-(FK)$_2$(FE)$_2$-NH$_2$.

Figure 3.8.S8. MALDI-TOF-MS data for Ac-KEFFFFKE-NH$_2$. 
Figure 3.8.S9. MALDI-TOF-MS data for Ac-(KFE)\(_2\)-NH\(_2\).

Figure 3.8.S10. MALDI-TOF-MS data for Ac-FFKEKEFF-NH\(_2\).
Table 3.8.S1. Calculated and observed m/z values for synthetic peptides (MALDI-TOF-MS).

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<td>1184.73</td>
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<tr>
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* calculated values
Figure 3.8.S11. Electron diffraction patterns for assembled peptides in water (pH 3–4). (A) Ac-(FKFE)$_2$-NH$_2$, 1.0 mM; (B) Ac-(FKFE)$_2$-NH$_2$, 0.2 mM; (C) Ac-(FK)$_2$(FE)$_2$-NH$_2$, 1.0 mM; (D) Ac-(FK)$_2$(FE)$_2$-NH$_2$, 0.2 mM; (E) Ac-KEFFFKFE-NH$_2$, 1.0 mM; (F) Ac-FFKEKEFF-NH$_2$, 1.0 mM.
Figure 3.8.S12. Calculated $d$-spacing values from electron diffraction patterns for assembled peptides in water (pH 3–4). (A) Ac-(FKFE)$_2$-NH$_2$, 1.0 mM; (B) Ac-(FKFE)$_2$-NH$_2$, 0.2 mM; (C) Ac-(FK)$_2$(FE)$_2$-NH$_2$, 1.0 mM; (D) Ac-(FK)$_2$(FE)$_2$-NH$_2$, 0.2 mM; (E) Ac-KEFFFFKE-NH$_2$, 1.0 mM; (F) Ac-FFKEKEFF-NH$_2$, 1.0 mM.
Figure 3.8.S13. Electron diffraction patterns for assembled peptides in buffered water at 1.0 mM concentrations (pH 7). (A) Ac-(FKFE)$_2$-NH$_2$; (B) Ac-(FK)$_2$(FE)$_2$-NH$_2$; (C) Ac-KEFFFFKE-NH$_2$; (D) Ac-FFKEKEFF-NH$_2$. 
Figure 3.8.S14. Calculated $d$-spacing values from electron diffraction patterns for assembled peptides in buffered water (pH 7). (A) Ac-(FKFE)$_2$-NH$_2$; (B) Ac-(FK)$_2$(FE)$_2$-NH$_2$; (C) Ac-KEFFFFKE-NH$_2$; (D) Ac-FFKEKEFF-NH$_2$. 

![Diffraction Patterns](image_url)
Chapter 4

4 Capacity for Increased Surface Area in the Hydrophobic Core of β-Sheet Peptide Bilayer Nanoribbons

4.1 Abstract

Amphipathic peptides with amino acids arranged in alternating patterns of hydrophobic and hydrophilic residues efficiently self-assemble into β-sheet bilayer nanoribbons. The hydrophobic side chain functionality is effectively buried in the interior of the putative bilayer of these nanoribbons. In this study, we investigated the consequence on self-assembly of increasing the surface area of side chain groups that
reside in the hydrophobic core of nanoribbons derived from Ac-(XKXE)\textsubscript{2}-NH\textsubscript{2} peptides (X = hydrophobic residue). A series of Ac-(XKXE)\textsubscript{2}-NH\textsubscript{2} peptides incorporating amino acids of increasing molecular volume and steric profile (X = phenylalanine (Phe), homophenylalanine (Hph), tryptophan (Trp), 1-naphthylalanine (1-Nal), 2-naphthylalanine (2-Nal), or biphenylalanine (Bip)) were assessed to determine the effect of these substitutions on self-assembly propensity and on the morphology of the resulting nanoribbon structures. Additional studies were conducted to determine the effects of incorporating amino acids with different steric profile in the hydrophobic core (Ac-XKFEFKFE-NH\textsubscript{2} and Ac-(XKFE)\textsubscript{2}-NH\textsubscript{2} peptides, X = Trp or Bip). Spectroscopic analysis by CD and FT-IR indicated β-sheet formation for all variants. Self-assembly rate increased with peptide hydrophobicity; increased molecular volume of the hydrophobic side chain groups did not appear to induce kinetic penalties on self-assembly rates. TEM imaging indicated variation in fibril morphology as a function of amino acid in the X positions. This study confirms that the hydrophobicity of amphipathic Ac-(XKXE)\textsubscript{2}-NH\textsubscript{2} peptides correlates to self-assembly propensity and that the hydrophobic core of the resulting nanoribbon bilayers have a significant capacity to accommodate sterically demanding functional groups. These findings provide insight that can be used to guide exploitation of self-assembled amphipathic peptides as functional biomaterials.

4.2 Introduction

Amphipathic peptides composed of alternating hydrophobic and hydrophilic amino acids have a strong propensity to self-assemble into amyloid-like β-sheet nanoribbons.\textsuperscript{1-3} This sequence pattern provides peptides in which the hydrophobic and hydrophilic side
chain groups are effectively segregated to opposite faces of the sequence in a $\beta$-strand orientation (Figure 1). This facilitates self-assembly into $\beta$-sheets that have hydrophobic and hydrophilic faces. Face to face lamination of two $\beta$-sheets effectively buries all hydrophobic functionality in the interior of the $\beta$-sheet bilayer, leaving the hydrophilic side chain groups exposed to the aqueous environment. This nanoribbon architecture provides polymer-like materials that are highly water soluble, facilitating use of these nanoribbons as materials for inventive applications that include ex vivo tissue engineering$^{4-7}$ and therapeutic drug delivery.$^{8-12}$ While many applications of self-assembled amphipathic peptide nanoribbons have been reported, fundamental questions regarding structure and function of these materials persist, limiting the ability to rationally design next-generation materials with precisely tuned emergent properties.

![Figure 4.1](image)

**Figure 4.1.** Schematic illustrating self-assembly of amphipathic Ac-(XKXE)$_2$-NH$_2$ peptides into $\beta$-sheet bilayer fibrils. The $\beta$-sheet bilayer axis is perpendicular to the page.

The hydrophobic functionality of amphipathic $\beta$-sheet peptides plays a critical role in driving self-assembly into $\beta$-sheet bilayer fibrils and also influences the emergent properties of the resulting materials.$^{13-18}$ We have conducted several studies in which self-assembly of Ac-(XKXE)$_2$-NH$_2$ and related Ac-(XK)$_4$-NH$_2$ peptides has been
characterized as a function of hydrophobic and aromatic character in the X position.\textsuperscript{15,19} Mutational analyses in which amino acids with varying hydrophobic and aromatic character (Ala, Val, Leu, Ile, Phe, cyclohexylalanine (Cha), and pentafluorophenylalanine (F\textsubscript{5}-Phe)) were incorporated in the X position of these peptides indicated that self-assembly propensity increased as the hydrophobicity of X increased. While aromatic character at X did not appear to strongly influence self-assembly propensity, peptides with aromatic residues did have unique fibril morphologies compared to nonaromatic peptides. At high peptide concentration, amphipathic peptide nanoribbons can entangle to form hydrogel networks; in our studies, peptides with aromatic residues in the X position exhibited enhanced mechanical rigidity of the resulting hydrogels relative to peptides with nonaromatic X residues. Thus, the amino acids that comprise the hydrophobic core of amphipathic peptide nanoribbons exert a strong influence on the properties of the assembled materials.

In order to more fully understand the relationship between the characteristics of the X residue in Ac-(XKXE)\textsubscript{2}-NH\textsubscript{2} peptides and the properties of the resulting self-assembled materials, we conducted an analysis of peptides that vary in exposed surface area of aromatic X side chain groups. The objective of these studies was to determine how side chain functionality with increasing molecular volume and steric profile would be tolerated within the hydrophobic core of β-sheet bilayer nanoribbons and what effect changing the exposed side chain surface area of the X residues would have on the resulting self-assembled materials. We assessed a series of Ac-(XKXE)\textsubscript{2}-NH\textsubscript{2} peptides in which amino acids of increasing side chain surface area were incorporated in the X position. Specifically, phenylalanine (Phe), homophenylalanine (Hph), tryptophan (Trp),
1-naphthylalanine (1-Nal), 2-naphthylalanine (2-Nal), and biphenylalanine (Bip) variants were prepared. It was found that each of these peptides successfully self-assembled, although some variation in nanoribbon morphology was observed. It was also found that the rate of self-assembly for these peptides increased as the hydrophobicity of the X residue increased.

In addition, the self-assembly behavior of Ac-(FKFE)$_2$-NH$_2$-derived peptides was also assessed as a function of replacement of one or two Phe residues (Ac-XKFEFKFE-NH$_2$ and Ac-(XKFE)$_2$-NH$_2$) with either Trp or Bip. In these studies, Trp and Bip represent a moderate and an extreme increase in side chain surface area and hydrophobicity (relative to Phe), respectively. Replacement of the $N$-terminal Phe residue (Phe 1) and a central Phe residue (Phe 5) was assessed due to the cross-strand pairing patterns of these specific residues in the $\beta$-sheet architecture. Phe 1 should be less sensitive to changes in molecular volume than Phe 5 since Phe 1 is unpaired in the proposed out of register antiparallel $\beta$-sheet nanoribbon structure, while Phe 5 is self-paired (Phe 5 side chains in neighboring $\beta$-strands within the $\beta$-sheet interact cross-strand, see Results and Discussion for a more detailed description). It was found that each of the Trp/Bip variants effectively formed $\beta$-sheet nanoribbon structures. The resulting nanoribbons exhibited subtle differences in morphology, ranging from flat nanoribbon structures to twisted ribbons.

Collectively, these studies indicate that amphipathic peptide self-assembly is sensitive to changes within the hydrophobic core of the putative $\beta$-sheet bilayer nanoribbons. Recent applications in which related amphipathic peptide nanoribbons were used to harbor hydrophobic drug-like molecules within the bilayer core for cellular delivery make this work of significant interest. $^{20}$ Insight into the steric packing tolerances of the
hydrophobic core of amphipathic peptide nanoribbons will facilitate elucidation of design principles for application of these materials in applications, including drug delivery, that require packaging of cargo in the interior of the bilayer.

4.3 Results and Discussion

4.3.1 Experimental rationale and peptide design

Amphipathic peptides, \((XZXZ)_n\), in which \(X\) residues are nonpolar and \(Z\) residues are polar have a high propensity to self-assemble into \(\beta\)-sheet bilayer nanoribbons. In order to more fully understand the relationship between the characteristics of the hydrophobic \(X\) residue in \(\text{Ac-(XKXE)}_2\cdot\text{NH}_2\) peptides and the properties of the resulting self-assembled materials, we conducted an analysis of peptides that vary in exposed surface area of the \(X\) side chain. Specifically, \(\text{Ac-(XKXE)}_2\cdot\text{NH}_2\) peptides in which all hydrophobic \(X\) residues were globally and systematically replaced with aromatic amino acids with increasing hydrophobicity and exposed molecular volume were prepared. Self-assembly of these peptides was characterized in order to assess the relationship between molecular volume/hydrophobicity of the core amino acids and self-assembly propensity as well as to gain insight into the tolerance for accommodation of increased steric bulk in the hydrophobic core of the putative nanoribbon bilayer.

A series of \(\text{Ac-(XKXE)}_2\cdot\text{NH}_2\) peptides in which amino acids of increasing side chain surface area were incorporated in the \(X\) position were assessed. Specifically, phenylalanine (Phe), homophenylalanine (Hph), tryptophan (Trp), 1-naphthylalanine (1-Nal), 2-naphthylalanine (2-Nal), or biphenylalanine (Bip) were substituted in the \(X\) position of \(\text{Ac-(XKXE)}_2\cdot\text{NH}_2\) peptides. These amino acids are listed in order of increasing hydrophobicity and molecular volume (Figure 4.2). Since previous work has
shown that the aromatic/nonaromatic character of X substituents in Ac-(XKXE)-NH₂ and related peptides exerts a dramatic effect on the assembly properties of these peptides, we opted to use only aromatic substituents in this study in order to remove aromaticity as a variable. Of the amino acids utilized herein, Phe is the least hydrophobic (Phe has a partition coefficient between water and octanol of 1.79 expressed relative to Gly, which is defined to have a partition coefficient of 0 between these two solvents) and has the smallest exposed volume of the substituents utilized herein (168.03 Å³). The additional methylene carbon in Hph results in a moderate increase in both hydrophobicity and volume relative to Phe (2.10 and 186.89 Å³, respectively). The indole side chain of Trp provides further increases in both hydrophobicity and exposed volume (2.25 and 202.39 Å³, respectively). The naphthyl side chains of 1-Nal and 2-Nal have similar hydrophobicity (3.08 and 3.15, respectively) and molecular volume (219.60 Å³ and 218.82 Å³, respectively) but differ in the spatial orientation of the side chain. Lastly, the 4,4′-bicyclic ring substitution of the Bip amino acid results in the greatest hydrophobicity (> 3.15) and exposed volume of the substituents considered herein (250.61 Å³). This collection of amino acid substituents represents a 1.5-fold increase in molecular volume (from smallest to largest) and a dramatic range of hydrophobic character that allows assessment of these variables on the self-assembly propensity of amphipathic peptides.
Figure 4.2. Structures of amino acids incorporated in the X position of Ac-(XKXE)$_2$-NH$_2$ variant peptides: phenylalanine (Phe), homophenylalanine (Hph), tryptophan (Trp), 1-naphthylalanine (1-Nal), 2-naphthylalanine (2-Nal), and biphenylalanine (Bip). Relative hydrophobicity is indicated in the form of partition coefficients ($\pi$) between octanol and water relative to glycine (defined as having a partition coefficient of 0 on this scale). Higher $\pi$ values correspond to higher hydrophobicity. The calculated van der Waals volume for each amino acid is also shown in order to indicate the degree of change in steric profile for each amino acid.

In addition to peptides in which the hydrophobic amino acids are globally replaced, a series of peptides with targeted substitutions were also explored. Structural models based on molecular dynamics simulations indicate that Ac-(FKFE)$_2$-NH$_2$ peptides adopt an out-of-register antiparallel $\beta$-sheet packing structure in the assembled bilayer nanoribbon fibrils (Figure 4.3). In these $\beta$-sheets, the $N$-terminal residue is out-of-
register, and thus unpaired in the context of bilayer nanoribbons. Thus, the side chain of
the N-terminal (position 1, \(X_1\)) is exposed to the aqueous environment and also has
unsatisfied hydrogen bond donor/acceptor groups. The remaining hydrophobic residue
side chains are paired and buried within the bilayer core of the resulting nanoribbons. We
hypothesized that substitution of Phe residues in the Ac-(FKFE)_2-NH\(_2\) peptide with either
Trp or Bip would have varying effects on self-assembly dependent on the position of
substitution. It was expected that substituting Phe 1 with either Trp or Bip (giving Ac-
XKFEFKFE-NH\(_2\) peptides) would be well tolerated since this residue (\(X_1\) in Figure 4.3)
is unpaired in the putative nanoribbon materials. Conversely, it was expected that
replacement of both Phe 1 and Phe 5 (giving Ac-(XKFE)_2-NH\(_2\) peptides in which \(X = \)
Trp or Bip; Phe 5 corresponds to \(X_2\) in Figure 4.3) would potentially alter the self-
assembly properties more dramatically since the \(X_2\) side chains are proposed to be paired
cross-strand in the nanoribbon bilayer sheets; substitutions at \(X_2\) should thus be more
sensitive to steric effects when the volume of this side chain is increased. Accordingly,
we also considered the self-assembly of Ac-XKFEFKFE-NH\(_2\) and Ac-(XKFE)_2-NH\(_2\)
variant peptides in which \(X\) was either Trp or Bip.
Figure 4.3. Proposed packing mode for antiparallel out-of-register amphipathic Ac-XKFEFKFE-NH₂ and Ac-(XKFE)₂-NH₂ peptides with Trp and Bip substitutions at position 1 (X₁) or at positions 1 and 5 (X₂). As shown schematically in this diagram, the position 1 substituents are unpaired cross-strand whereas the position 5 substituents are self-paired.

Peptides were synthesized using standard Fmoc solid phase peptide synthesis methods as N-terminal acetyl and C-terminal amide sequences, and the relative hydrophobicity of each peptide was characterized. Details for peptide synthesis, purification, and characterization are discussed in the Experimental Section (characterization data is shown in the Supporting Information, Figures 4.8.S1–S20 and Table 4.8.S1). Relative peptide hydrophobicity was estimated by high-performance liquid chromatography (HPLC) analysis. A comparison of HPLC retention times under identical stationary and mobile phase conditions gives reasonable estimates of relative hydrophobicity: longer retention times correspond to higher hydrophobicity with reverse phase stationary phase materials. The HPLC retention times for each Ac-(XKXE)₂-NH₂ peptide are shown in Table 4.1 (peptides 1–6). As expected, the Phe-containing peptide...
had the earliest retention time of 12.50 min. However, Ac-(WKWE)₂-NH₂ had an earlier retention time (12.83 min) than the Ac-(HphKHphE)₂-NH₂ peptide (13.58 min), which would not be predicted based on the literature hydrophobicity coefficients for the constituent amino acids of these peptides. This earlier retention time for Trp is possibly due to the hydrogen bonding capabilities of the indole side chain that may offset the increased side chain hydrophobicity. HPLC analysis indicated that the Ac-(1-NalK₁-NalE)₂-NH₂ and Ac-(2-NalK₂-NalE)₂-NH₂ peptides had identical retention times (14.32 min). The Bip amino acid does not have a reported hydrophobicity coefficient, but, as expected, the Ac-(BipKBipE)₂-NH₂ peptide is the most hydrophobic of the series (retention time of 15.00 min). Thus, the order of hydrophobicity of the Ac-(XKXE)₂-NH₂ peptides studied herein increases in the order: X = Phe < Trp < Hph < 1-Nal = 2-Nal < Bip.

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<th>Sequence</th>
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</tbody>
</table>
The hydrophocity of the Ac-XKFEFKFE-NH$_2$ and Ac-(XKFE)$_2$-NH$_2$ peptides (X = Trp or Bip) was also assessed by HPLC analysis (peptides 7–10, Table 4.1). It was found that the Ac-WKFEFKFE-NH$_2$ and Ac-(WKFE)$_2$-NH$_2$ peptides had similar retention times of 12.68 and 12.65 min, respectively. As a comparison, the Ac-(FKFE)$_2$-NH$_2$ and Ac-(WKWE)$_2$-NH$_2$ peptides had similar retention times of 12.50 and 12.83 min, respectively. The Ac-BipKFEFKFE-NH$_2$ (13.19 min) and Ac-(BipKFE)$_2$-NH$_2$ (13.68 min) peptides were significantly more hydrophobic than the parent Ac-(FKFE)$_2$-NH$_2$ peptide, but less hydrophobic than the Ac-(BipKBipE)$_2$-NH$_2$ peptide (15.00 min). Thus, this series of mono- and di-substituted variants had a narrower range of hydrophobicity than the global variants (peptides 1–6, Table 1).

4.3.2 Characterization of Ac-(XKXE)$_2$-NH$_2$ variant peptide self-assembly.

Self-assembly of each peptide was initiated by dissolution of the lyophilized peptide in 5% HFIP and water at 0.2 mM. The increased hydrophobicity of some of the variants, particularly 1- and 2-Nal and Bip-containing peptides, resulted in reduced solubility in water. It was found however, that the addition of a small amount of hexafluoroisopropanol (HFIP, 5% by volume) to self-assembly solutions effectively solubilized all peptides (peptides were initially dissolved in HFIP to inhibit $\beta$-sheet self-assembly, then diluted to the desired concentration in water).$^{15,23-25}$ Previous studies on the self-assembly of Ac-(XKXE)-NH$_2$ peptides in water indicate that self-assembly of these peptides occurs very rapidly. As described below, the inclusion of HFIP as a cosolvent (even 5% v/v) has an inhibitory effect on self-assembly of amphipathic peptides. Thus,
self-assembly was monitored over a period of 24 hours to ensure complete assembly of each peptide studied herein.

Self-assembly of the peptides reported herein was initially assessed as a function of evolution of β-sheet peptide secondary using circular dichroism (CD) and Fourier transform infrared (FT-IR) spectroscopic techniques.\textsuperscript{1,26-32} CD spectra were recorded immediately after sample preparation to monitor formation of β-sheet secondary structure over a span of 24 hours at time points of 0, 1, 2, 4, 12, and 24 hours. FT-IR spectra were recorded after the peptides were allowed to assemble for 24 hours.

A CD spectrum that is characteristic of β-sheet secondary structure features a minimum between 215–220 nm and a maximum from 190–200 nm.\textsuperscript{33,34} In this study, CD spectra for these amphipathic β-sheet peptides were often found to deviate dramatically from canonical β-sheet spectra. For example, as shown previously, the Ac-(FKFE)$_2$-NH$_2$ peptide has a strong minimum at 205 nm in addition to the classical minimum at 218 nm and maximum at 195 nm (Figure 4.4A).\textsuperscript{15,19,35-37} The minimum at 205 nm has been attributed to electronic effects from π-stacking of aromatic side chains in the hydrophobic core\textsuperscript{38} or to distortion of the assembled β-sheets.\textsuperscript{39} In an aqueous environment, a strong β-sheet CD signal consistent with complete assembly into β-sheet nanoribbons is typically detected within minutes for Ac-(FKFE)$_2$-NH$_2$. However, in this study, the HFIP cosolvent slows self-assembly as evidenced by an intensification of the CD signal over a 24-hour time period (Figure 4.4A).\textsuperscript{15,23}

The minute change of an additional β-alkyl carbon in the Hph residue resulted in a significantly different CD spectrum for the Ac-(HphKHphE)$_2$-NH$_2$ variant (Figure 4.4B). A minimum at 220 nm and maximum at 195 nm corresponds to classical β-sheet
structure. The lack of signal at ~205 nm for the Ac-(HphKHphE)$_2$-NH$_2$ peptide implies that $\pi$-stacking or $\beta$-sheet distortion$^{39}$ is dramatically altered relative to the Ac-(FKFE)$_2$-NH$_2$ peptide. The additional degrees of freedom afforded by the additional methylene carbon in the Ac-(HphKHphE)$_2$-NH$_2$ peptide, thus has fairly dramatic effects on $\beta$-sheet packing during self-assembly. This is also evident since the $\beta$-sheet signal is essentially unchanged over 24 hours even in the presence of the HFIP co-solvent, indicating that the kinetics of self-assembly are dramatically accelerated for the Ac-(HphKHphE)$_2$-NH$_2$ peptide. CD analysis thus suggests that increasing the length of the Phe side chain by addition of one alkyl carbon potentially stabilizes $\beta$-sheet formation for the amphipathic Ac-(HphKHphE)$_2$-NH$_2$ peptide relative to the parent Ac-(FKFE)$_2$-NH$_2$.

**Figure 4.4.** CD spectra in 5% HFIP and water at time periods of 0, 1, 2, 4, 12, and 24 h for amphipathic Ac-(XKXE)$_2$-NH$_2$ peptides in which X is (A) Phe; (B) Hph; (C) Trp; (D) 1-Nal; (E) 2-Nal; (F) Bip.
The CD spectrum for Ac-(WKWE)$_2$-NH$_2$ diverges dramatically from canonical $\beta$-sheet patterns, although it is similar to that previously reported for the (WKWE)$_2$W-NH$_2$ peptide that has been described as $\beta$-sheet.$^{40}$ Initially, the CD spectrum had a broad minimum at 210 nm that possibly reflects a mixture of $\alpha$-helix and $\beta$-sheet structures due to the inhibitory effect of the HFIP cosolvent on self-assembly (Figure 4.4C).$^{41-44}$ Over time, the signal evolved to feature a minimum at 208 nm (possibly an amide n $\rightarrow$ $\pi^*$ transition) and a maximum at 220 nm (possibly $\pi$ $\rightarrow$ $\pi^*$ transition of interacting indole functionality).$^{45-47}$ These CD patterns are similar to those previously observed in Trp-containing $\beta$-hairpin peptides and are indicative of a strong negative–positive exciton-coupled band between Trp-Trp chromophores within the $\beta$-hairpin.$^{48-52}$ Thus, CD spectrum of the Ac-(WKWE)$_2$-NH$_2$ peptide is consistent with an assembled state in which Trp residues interact. Since electronic effects from the Trp residue dominate these spectra, $\beta$-sheet structure requires validation using complementary FT-IR analysis (discussed below). However, it is interesting that the Ac-(WKWE)$_2$-NH$_2$ peptide appears to exhibit assembly behavior that is kinetically more similar to that of Ac-(FKFE)$_2$-NH$_2$ (occurring over 24 hours) that to that of Ac-(HphKHphE)$_2$-NH$_2$, despite the larger molecular volume and hydrophobicity of Trp relative to Hph. The effect of a change in position of the Hph side chain by a single carbon unit appears to be even more dramatic in this light.

The 1-Nal and 2-Nal-containing peptides also exhibited CD spectra that diverge from classical $\beta$-sheet secondary structures. However, the large minimum at 230 nm for Ac-(1-NalK1-NalE)$_2$-NH$_2$ is indicative of aromatic stacking between 1-naphthylalanine
side chains (Figure 4.4D). A blue shift is often observed in the CD signal for 2-naphthyalanine; this was apparent in Ac-(2-NalK2-NalE)2-NH2 that had a minimum at ~210 nm with a maximum at 230 nm. The HFIP cosolvent did not appear to influence the kinetics of self-assembly for these peptides, since no change in signal intensity was observed over 24 hours. CD spectra of the 1- and 2-Nal peptides are dominated by electronic effects from interaction of the naphthyl side chains, making assessment of β-sheet structure difficult without confirmation by other spectroscopic methods (see FT-IR analysis below).

Lastly, the Ac-(BipKBipE)2-NH2 peptide had a unique saddle-like CD signature. It had two minima at 202 nm and 222 nm, consistent with the characteristic Cotton effect for biphenyl-containing compounds. Electronic effects from the biphenyl side chain dominate the spectrum and suggest self-assembly, although the β-sheet structure of these assemblies requires additional spectroscopic analysis (as described below). The HFIP cosolvent had no effect on evolution of signal intensity over time, suggesting that this peptide (as well as the Hph, 1-Nal, and 2-Nal peptides) are sufficiently hydrophobic (or possess other unique packing effects) to enable rapid assembly even in the presence of organic cosolvents with disaggregant properties.

Based on CD analysis only two peptides, Ac-(FKFE)2-NH2 and Ac-(HphKHpHE)2-NH2, are clearly self-assembled into β-sheet structures, although spectra of the other peptides suggest that self-assembly has occurred. The Trp, 1-Nal, 2-Nal, and Bip-containing peptides have CD spectra that are dominated by electronic effects from the aromatic side chain groups, making definitive assessment of β-sheet structure impossible by CD spectroscopic analysis alone. Therefore, FT-IR analysis was used in
parallel to assess the secondary structure for putative assemblies of each variant peptide (Figure 4.5). $\beta$-sheet peptides have a characteristic amide I stretch between 1615 and 1635 cm$^{-1}$. As previously reported, the Ac-(FKFE)$_2$-NH$_2$ peptide has an amide I stretch at 1618 cm$^{-1}$, consistent with $\beta$-sheet self-assembly (Figure 4.5). Each of the other peptides assessed herein also had FT-IR spectra consistent with $\beta$-sheet self-assembly, with amide I stretches from 1616–1620 cm$^{-1}$. Thus, FT-IR analysis confirms that each Ac-(XKXE)$_2$-NH$_2$ variant, regardless of side chain volume or hydrophobicity, effectively forms $\beta$-sheet structures.

Figure 4.5. FT-IR spectra for Ac-(XKXE)$_2$-NH$_2$ peptides in which X = Phe, Hph, Trp, 1-Nal, 2-Nal, or Bip. Each peptide exhibits a characteristic amide I signal at ~1618 cm$^{-1}$ indicating $\beta$-sheet secondary structure for all variants.
4.3.3 TEM analysis of nanoribbon morphology for Ac-(XKXE)$_2$-NH$_2$ peptides

CD and FT-IR are useful tools for characterizing secondary structure that cannot, however, independently confirm the presence of self-assembled $\beta$-sheet structures. Imaging methods such as transmission electron microscopy (TEM) must be used to confirm assembly of peptides into higher order nanoribbon/fibril materials and to characterize the morphology of these materials. Accordingly, TEM analysis was used to characterize the self-assembled materials derived from Ac-(XKXE)$_2$-NH$_2$ peptides.

As previously reported, the Ac-(FKFE)$_2$-NH$_2$ peptide assembled into nanoribbon fibrils.$^{15,37,61,62}$ Left-handed helical nanoribbons (pitch of 19.1 ± 1.3 nm) were observed to coexist with flat nanoribbons (Figure 4.6A); both morphologies had an average ribbon diameter of 8.8 ± 0.8 nm. The helical nanoribbons dominate solutions at early timepoints (minutes to hours) with the flat nanoribbons becoming dominant after extended incubation periods. While detailed understanding for evolution of helical ribbons into flat ribbons is lacking, it can be presumed that the helical structures represent kinetic products whereas the flat nanoribbons are thermodynamic products.$^{61,62}$
Figure 4.6. TEM images of Ac-(XKXE)$_2$-NH$_2$ peptide nanoribbons. (A) $X = $ Phe; (B) $X = $ Hph; (C) $X = $ Trp; (D) $X = $ 1-Nal; (E) $X = $ 2-Nal; (F) $X = $ Bip.

Assemblies of variant Ac-(XKXE)$_2$-NH$_2$ peptides display subtle morphological differences relative to assemblies of the parent Ac-(FKFE)$_2$-NH$_2$ peptide. Ac-(HphKHphE)$_2$-NH$_2$ assemblies (Figure 4.6B) appeared as 7.6 ± 1.3 nm diameter nanoribbon fibrils that lacked the regular helical structure observed at early timepoints with the Ac-(FKFE)$_2$-NH$_2$ peptide. Instead, Ac-(HphKHphE)$_2$-NH$_2$ nanoribbons were either flat or gently twisting, without any regularity in the pitch or frequency of the twist. Ac-(WKWE)$_2$-NH$_2$ fibrils were predominately flat nanoribbons with a diameter of 7.3 ± 1.6 nm (Figure 4.6C). The TEM images for the 1-Nal variant displayed densely packed fibrils that were primarily flat loosely coiled nanoribbons with diameters of 8.2 ± 1.2 nm (Figure 4.6D). The 2-Nal peptides assembled into nanoribbons with similar morphology
to the 1-Nal peptides, with diameters of 8.9 ± 1.1 nm (Figure 4.6E). Lastly, Ac-(BipKBipE)2-NH2 assembled into densely packed helical nanoribbons that were significantly thinner (5.5 ± 0.9 nm) than the other variants (Figure 4.6F).

The reason for this subtle variation in fibril morphology as a function of the identity of the hydrophobic residues in Ac-(XKXE)2-NH2 peptides is not clearly understood. However, the fact that morphology does vary, even subtly, implies that the volume and orientation of the side chain groups in the hydrophobic bilayer of these assembled materials does exert an influence on peptide packing within the β-sheet that is manifested in the overall structure of the assembled material. Presumably, side chain packing can directly influence nanoribbon morphology directly as a function of volume in the bilayer interior, and indirectly by subtle perturbation of the β-strand twist for the constituent peptides within the β-sheet structures. Molecular volume and steric profile of the hydrophobic amino acids in self-assembling amphipathic β-sheet peptides is thus a variable that can potentially be used to tune the emergent properties of the assembled materials.

4.3.4 Self-assembly of mono- and di-substituted Ac-XKFEFKFE-NH2 and Ac-(XKFE)2-NH2 variants.

The effect of steric factors on the self-assembly of Ac-(FKFE)2-NH2-derived peptides was also assessed by more subtle mono- and di-substituted Phe 1 and Phe 1/Phe 5 variants. Ac-XKFEFKFE-NH2 and Ac-(XKFE)2-NH2 peptides in which X is either Trp or Bip were prepared for these studies (Table 1, peptides 7–10). The positions for these substitutions were chosen due to the differences these positions have on packing order based on the putative structure of the β-sheet materials. The Phe 1 side chain is
presumably unpaired whereas the Phe 5 side chain potentially self-pairs cross-strand within the $\beta$-sheet. Thus, substitutions at Phe 5 should exert a more dramatic effect on self-assembly than substitutions at position 1. In order to maintain consistency with the assessment of peptides 1–6, self-assembly of peptides 7–10 was also assessed in 5% HFIP/water (v/v). Substitutions with Trp and Bip were explored to assess the affect of both subtle and extreme changes in molecular volume and hydrophobicity.

The mono-substituted Ac-WKFEFKFE-NH$_2$ peptide effectively self-assembled into $\beta$-sheet materials as evidenced by CD and FT-IR analysis (Figure 4.7A). The CD spectrum for this peptide was similar to that of Ac-(FKFE)$_2$-NH$_2$ $\beta$-sheet nanoribbons, with minima at 205 and 220 nm with a maximum at 195 nm. As with the Ac-(FKFE)$_2$-NH$_2$ peptide, the HFIP cosolvent inhibited the kinetics of self-assembly, which required ~24 hours to reach equilibrium. FT-IR analysis confirms that Ac-WKFEFKFE-NH$_2$ forms $\beta$-sheet structured materials (Figure 4.7C). Thus, spectral evidence suggests that Phe 1 → Trp substitution has only minimal impact on the self-assembly of this variant.

**Figure 4.7.** (A) CD spectra of Ac-WKFEFKFE-NH$_2$ in 5% HFIP/water over 24 hours; (B) CD spectra for Ac-(WKFE)$_2$-NH$_2$ in 5% HFIP/water over 24 h; (C) FT-IR spectra for Ac-BipKFEFKFE-NH$_2$ and Ac-(BipKFE)$_2$-NH$_2$ (24 h, 5% d$_2$-HFIP/D$_2$O).
The di-substituted Ac-(WKFE)$_2$-NH$_2$ variant exhibited a slightly modified CD pattern likely due to more extensive electronic effects from the cross-strand interaction of Trp 5 residues within the hydrophobic bilayer (Figure 4.7B). The CD spectrum of Ac-(WKFE)$_2$-NH$_2$ featured prominent minima at 202 and 218 nm that intensified over 24 hours, similar to the Ac-(WKWE)$_2$-NH$_2$ peptide (Figure 4.4C). The second, less prominent, minimum at 220 nm in the CD spectrum of Ac-(WKFE)$_2$-NH$_2$, replaced the maximum in this position in the CD spectrum for Ac-(WKWE)$_2$-NH$_2$. The FT-IR spectrum for Ac-(WKFE)$_2$-NH$_2$ was consistent with $\beta$-sheet structure, with an amide I stretch at 1620 cm$^{-1}$ (Figure 4.7C). It was observed that the doubly substituted Ac-(WKFE)$_2$-NH$_2$ exhibited a more dramatic intensification of CD signal over time relative to the monosubstituted Ac-WKFEFKFE-NH$_2$ peptide, consistent with a more dramatic effect on self-assembly by substitutions that fall within the core of the bilayer as opposed to the edge of the bilayer.

Bip substitution had a more dramatic effect on self-assembly of Ac-(FKFE)$_2$-NH$_2$-derived peptides than did Trp substitution. Phe 1 $\rightarrow$ Bip substitution (Ac-BipKFEFKFE-NH$_2$) provided a peptide that, similar to the parent peptide and to Ac-WKFEFKFE-NH$_2$, had a CD spectrum with minima at 202 and 218 nm that intensified dramatically over 24 hours (Figure 4.8A). The magnitude of this change was 10-fold greater for Ac-BipKFEFKFE-NH$_2$ than was observed for either Ac-(FKFE)$_2$-NH$_2$ or Ac-WKFEFKFE-NH$_2$. Thus, dramatic changes in the side chain steric profile at position 1 in these amphipathic sequences can significantly alter the kinetics of self-assembly even though this side is presumably unpaired in the context of assembled nanoribbons. The
FT-IR spectrum for Ac-BipKFEFKFE-NH₂ indicated an amide I stretch at 1618 cm⁻¹, consistent with β-sheet self-assembly.

**Figure 4.8.** CD and FT-IR spectra for the mono- and di-substituted Bip variants (0.2 mM peptide). (A) CD spectra of Ac-BipKFEFKFE-NH₂ in 5% HFIP/water over 24 hours; (B) CD spectra for Ac-(BipKFE)₂-NH₂ in 5% HFIP/water over 24 h; (C) FT-IR spectra for Ac-BipKFEFKFE-NH₂ and Ac-(BipKFE)₂-NH₂ (24 h, 5% d₂-HFIP/D₂O).

Incorporating a second internal Bip substitution at Phe 5 (Ac-(BipKFE)₂-NH₂) again altered the self-assembly of this peptide relative to the parent peptide and the monosubstituted Bip 1 variant. The CD spectrum for the Ac-(BipKFE)₂-NH₂ peptide initially featured only a single minimum at 218 nm; over a period of 24 hours a second prominent minimum at 205 nm (attributed to π-stacking of the Phe side chains) was also observed. This suggests that rearrangement of the packing orientation of the side chain groups can occur within the bilayer core over time. The increased hydrophobicity of the Ac-(BipKFE)₂-NH₂ peptide increased the kinetics of self-assembly, with only minor intensification of the CD spectral signals over time (Figure 4.8B). Unlike the previously described mono- and di-substituted variants, the Ac-(BipKFE)₂-NH₂ CD signal was seemingly unaffected by the HFIP cosolvent. The FT-IR spectrum confirmed β-sheet structure with an amide I stretch at 1620 cm⁻¹ (Figure 4.8C).
TEM images of the mono- and di-substituted peptides were obtained to confirm self-assembly and to assess the fibril morphology of the β-sheet structures. The fibril morphology for Ac-WKFEFKFE-NH₂ was very similar to Ac-(FKFE)₂-NH₂ (see Figure 4.6A for comparison). In addition to helical nanoribbons that were ~8 nm in diameter, however, thinner, twisted nanoribbons that were 5.3 ± 1.5 nm wide were also observed (Figure 4.9A). The Ac-(WKFE)₂-NH₂ peptide had a combination of twisted helical nanoribbons and flat nanotapes with widths averaging 7.3 ± 1.5 nm (Figure 4.9B). It is evident by these changes in fibril morphology that location of the increased side chain surface area influences the packing mode for β-sheet fibrils since the N-terminal substitution had relatively minimal effects compared to the internal substitutions at Phe 5.

TEM images of the mono- and di-substituted peptides were obtained to confirm self-assembly and to assess the fibril morphology of the β-sheet structures. The fibril morphology for Ac-WKFEFKFE-NH₂ was very similar to Ac-(FKFE)₂-NH₂ (see Figure 4.6A for comparison). In addition to helical nanoribbons that were ~8 nm in diameter, however, thinner, twisted nanoribbons that were 2.8 ± 0.3 nm wide were also observed (Figure 4.9A). The Ac-(WKFE)₂-NH₂ peptide had a combination of twisted helical nanoribbons and flat nanotapes with widths averaging 8.06 ± 0.7 nm and 8.77 ± 0.8 nm respectively (Figure 4.9B). It is evident by these changes in fibril morphology that location of the increased side chain surface area influences the packing mode for β-sheet fibrils since the N-terminal substitution had relatively minimal effects compared to the internal substitutions at Phe 5.

The fibril morphologies for the Bip variants differed more dramatically from the parent peptide. Images of the Ac-BipKFEFKFE-NH₂ peptide indicated unique bundling
of the fibrils that were $2.9 \pm 0.5$ nm in diameter, roughly corresponding to the width of a single $\beta$-strand peptide (Figure 4.9C). These thinner fibril types may result from blockage of edge-to-edge alignment of $\beta$-sheets that must account for nanotape widening by the sterically demanding Bip residue that is at the exposed edge of the putative antiparallel $\beta$-sheet. Interestingly, the high degree of bundling of these fibrils may also be due to exposure of the hydrophobic Bip residue; bundling may occur in order to desolvate this functionality. Lastly, the Ac-(BipKFE)$_2$-NH$_2$ peptide assembled into several morphologies ranging from very thin ribbons averaging $3.2 \pm 0.3$ nm in diameter to wider nanoribbons averaging $10.0 \pm 1.1$ nm in diameter (Figure 4.9D). Changes in $\beta$-sheet packing due to incorporation of a single internal Bip residue apparently facilitates self-assembly into wider nanoribbons. Muller et al. observed a similar phenomenon when aliphatic residues of varying steric bulk were seen to alter the morphology of self-assembled materials from amyloid-inspired peptides.$^{39}$

Changes in fibril morphology as a function of mono- or disubstitution of Phe with more sterically demanding residues is clearly sensitive to the position of substitution. Introducing steric bulk within the center of Ac-(FKFE)$_2$-NH$_2$ $\beta$-strands has a more dramatic overall effect on assembled nanoribbon morphology than introduction of steric bulk at the exposed $N$-terminal residues. The comparative morphologies for the self-assembled Ac-WKFEFKFE-NH$_2$ and Ac-BipKFEFKFE-NH$_2$ peptides were similar. Conversely, the morphologies of self-assembled materials derived from the Ac-(WKFE)$_2$-NH$_2$ and Ac-(BipKFE)$_2$-NH$_2$ peptides were dramatically different. This indicates that changes in positions in which cross-strand pairing within the $\beta$-sheet structure is effected are highly influential on emergent nanoribbon structure. Conversely,
the exposed N-terminal residue presumably does not participate in cross-strand pairing between β-strands but rather functions to mediate interactions between β-sheets, which exerts more subtle effects on overall fibril structure.

Figure 4.9. TEM images for the mono- and di-substituted assembled materials (A) Ac-WKFEFKFE-NH$_2$ (B) Ac-(WKFE$_2$)-NH$_2$ (C) Ac-BipKFEFKFE-NH$_2$ (D) Ac-(BipKFE$_2$)-NH$_2$. 
4.4 Discussion

Changes to the aromatic surface area and hydrophobicity of X residues in amphipathic Ac-(XKXE)$_2$-NH$_2$ peptides can dramatically influence rates of self-assembly and the morphology of the resulting materials. As described above, it is significant that the hydrophobic core of these putative bilayer materials can accommodate large changes in molecular volume. This provides meaningful insight into the potential of these materials for applications in which a large variety of hydrophobic groups may be required in these positions. For example, one could envision the inclusion of bulky chromophores in this hydrophobic core for the design of light harvesting materials. The morphology of the assembled structures is also sensitive to changes in the steric profile of the constituent hydrophobic amino acids. Molecular volume can thus be exploited as a variable to tune the emergent structure of the resulting self-assembled materials. Changes in morphology do not rely on global changes to all hydrophobic residues in these amphipathic peptides; even single amino acid substitutions can influence fibril morphology. Finally, these studies further confirm that increased in amphipathic peptide hydrophobicity correlate to enhancements in the rates of assembly for these types of materials.

While these studies provide significant insight into the role of molecular volume and hydrophobicity on the self-assembly of amphipathic peptides, they also raise interesting questions. It has been empirically established that changes in molecular volume at X in Ac-(XKXE)$_2$-NH$_2$ and related peptides correlate to changes in the morphology of the assembled material. The molecular basis for these changes, however, is not understood. These observations emphasize a critical need for higher resolution structural analysis of materials derived from self-assembled amphipathic peptides. Solid-
state NMR has been of critical value in the study of amyloid systems\textsuperscript{53} and greater application of these tools to self-assembled amphipathic materials\textsuperscript{64} is warranted and needed to facilitate a shift in research in this area from empirical approaches to rational, structure-based approaches.

4.5 Conclusion

Amphipathic peptide self-assembly is sensitive to changes within the hydrophobic core of the putative $\beta$-sheet bilayer nanoribbons. Herein, we have reported that Ac-(XKXE)$_2$-NH$_2$ peptides with hydrophobic amino acids of increasing surface area and hydrophobicity ($X = \text{Phe, Hph, Trp, 1-Nal, 2-Nal, or Bip}$) effectively self-assemble in all cases, demonstrating that the hydrophobic core of the resulting bilayer nanoribbons has a great capacity to accommodate packing of even very large side chain groups. Interestingly, subtle differences in morphology were observed as a function of hydrophobic $X$ residues. This suggests that aromatic/hydrophobic packing effects can also elicit changes in $\beta$-sheet packing (presumably via strand registry or strand twisting) that give rise to the observed variation in nanoribbon appearance. In addition, these studies also confirm that self-assembly kinetics is accelerated as the hydrophobicity of the core $X$ amino acids increases. These studies clarify the structural tolerances of amphipathic $\beta$-sheet peptide self-assembly and provide insight into the capacity for appending cargo into the hydrophobic core of these self-assembled nanoribbon architectures. Future studies will focus on evaluation of the molecular basis for the variation in nanoribbon structural morphology as the structure of the core hydrophobic $X$
residues are altered and on understanding how these changes relate to the emergent properties of the self-assembled materials.

4.6 Experimental Procedures

4.6.1 Peptide Synthesis, Purification, and Characterization

Peptides were synthesized on a microwave-equipped Liberty Peptide Synthesizer (CEM®) using standard Fmoc protection and HBTU/HOBt activation chemistry. Rink amide resin (Advanced ChemTech, 100-200 mesh, 0.2 mmol g⁻¹) was used as the solid support to provide C-terminal amide peptides. Peptides were treated with 20% acetic anhydride in DMF to give N-terminal acetyl sequences. Side chain-deprotection and cleavage from the solid support was accomplished by treatment with trifluoroacetic acid (TFA), triisopropylsilane (TIS), and water (95:2.5:2.5, v/v/v) (room temperature, 1 h). Cleavage solutions were concentrated to 10% of the reaction volume after which peptides were precipitated in ethyl ether and isolated by centrifugation; precipitated peptides were washed by resuspension ether. The isolated solid peptide was then dissolved in DMSO for purification by high-performance liquid chromatography (HPLC).

Purification of synthetic peptides was conducted using a Shimadzu LC-AD HPLC instrument over a reverse phase C18 stationary phase (Waters, BEH300, 10 µm, 19 × 250 mm). A binary gradient of acetonitrile and water with 0.1% TFA at 10 mL min⁻¹ was used as the mobile phase and eluent was monitored by UV absorbance at 215 and 254 nm. Fractions were collected and lyophilized and then analyzed by analytical HPLC (reverse phase C18, Waters, BEH300, 10 µm, 4.6 × 250 mm) to confirm purity (Supporting Information, Figures 4.8.S1–S10). Peptide identity was confirmed by MALDI-TOF mass spectroscopy (Supporting Information, Figures 4.8.S11–S20). Ac-
(FKFE)$_2$-NH$_2$ m/z 1162.28 (1162.34 calcd for [MH]$^+$), m/z 1185.25 (1185.32 calcd for [MNa]$^+$), m/z 1201.20 (1201.43 calcd for [MK]$^+$). Ac-(HphKHphE)$_2$-NH$_2$ m/z 1219.58 (1219.45 calcd for [MH]$^+$), m/z 1241.57 (1241.43 calcd for [MNa]$^+$), m/z 1257.55 (1257.54 calcd for [MK]$^+$). Ac-(WKWE)$_2$-NH$_2$ m/z 1319.51 (1319.49 calcd for [MH]$^+$), m/z 1341.50 (1341.47 calcd for [MNa]$^+$), m/z 1357.55 (1357.58 calcd for [MK]$^+$). Ac-(1-NalK1-Nal-E)$_2$-NH$_2$ m/z 1363.47 (1363.58 calcd for [MH]$^+$), m/z 1385.46 (1385.56 calcd for [MNa]$^+$), m/z 1401.43 (1401.67 calcd for [MK]$^+$). Ac-(2-NalK2-Nal-E)$_2$-NH$_2$ m/z 1363.54 (1363.58 calcd for [MH]$^+$), m/z 1385.51 (1385.56 calcd for [MNa]$^+$), m/z 1401.47 (1401.67 calcd for [MK]$^+$). Ac-(BipKBEFKE-E)$_2$-NH$_2$ m/z 1467.17 (1467.73 calcd for [MH]$^+$), m/z 1489.16 (1489.71 calcd for [MNa]$^+$), m/z 1505.13 (1505.82 calcd for [MK]$^+$). Ac-WKFEFKFE-NH$_2$ m/z 1202.85 (1202.38 calcd for [MH]$^+$), m/z 1224.85 (1224.36 calcd for [MNa]$^+$), m/z 1240.83 (1240.47 calcd for [MK]$^+$). Ac-(WKFE)$_2$-NH$_2$ m/z 1241.78 (1241.42 calcd for [MH]$^+$), m/z 1263.49 (1263.40 calcd for [MNa]$^+$), m/z 1279.47 (1279.51 calcd for [MK]$^+$). Ac-BipKFEFKFE-NH$_2$ m/z 1239.59 (1239.44 calcd for [MH]$^+$), m/z 1261.53 (1261.42 calcd for [MNa]$^+$), m/z 1277.51 (1277.53 calcd for [MK]$^+$). Ac-(BipKFE)$_2$-NH$_2$ m/z 1314.87 (1315.54 calcd for [MH]$^+$), m/z 1336.85 (1337.52 calcd for [MNa]$^+$), m/z 1352.81 (1353.63 calcd for [MK]$^+$). See Figure 4.9.S1-S20 in SI and Table 4.9.S1 for HPLC and MALDI-MS data.

4.6.2 Peptide self-assembly

Peptide self-assembly was initiated in a solution of water and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (95:5, v/v) (pH 3-4 as a consequence of residual TFA from HPLC purification). Self-assembly was analyzed at a peptide concentration of 0.2 mM quantified according to previously described protocols. Lyophilized peptides
were dissolved in an acetonitrile:water:TFA mixture (60:39.9:0.1, v/v/v) to maintain an unassembled peptide state. Peptide concentrations were determined from these solutions by analytical HPLC analysis and correlation of HPLC peak area to a standard curve constructed for each peptide (see Supporting Information, Figures 4.8.S21-S28 for standard curves). Standard concentration curves were prepared as described previously and integrated peak areas were correlated to absolute peptide concentration by amino acid analysis (AIBiotech, Richmond, VA). Aliquots of the desired peptide quantity were frozen, lyophilized, and used immediately in self-assembly studies. Self-assembly was initiated by dissolving lyophilized peptides in HFIP followed by addition of water to give a peptide concentration of 0.2 mM (final solvent composition of 5% HFIP by volume); solutions were treated by vortex (1 min) and sonication (5 min) to give optically transparent, homogenous solutions. Self-assembly was characterized using circular dichroism (CD), Fourier transform infrared (IR) spectroscopy, and transmission electron microscopy (TEM) imaging as described in the following sections.

4.6.3 Circular Dichroism Spectroscopy

CD spectra were recorded on an AVIV 202 circular dichroism spectrometer. Spectra were obtained using a 0.1 mm path length quartz cuvette (Hellma) at 25 °C from 260 to 190 nm with a 1.0 nm step, 1.0 nm bandwidth, and a 3 s collection time per step. Spectra were collected at 0, 1, 2, 4, 12, and 24 h for each sample. Background subtraction with 5% HFIP/water, conversion to molar ellipticity, and data smoothing with a least squares fit was performed using the AVIV software.
4.6.4 Fourier Transform Infrared Microscopy

FT-IR spectra were obtained using a Shimadzu 8400 FT-IR spectrometer. Prior to FT-IR analysis, TFA counter-ions were removed from the purified peptides through anion exchange by dissolving the peptides in 40% $d_6$-acetonitrile/D$_2$O (v/v) with 1% DCl followed by lyophilization. The lyophilized peptides were dissolved in 5% $d_2$-HFIP/D$_2$O with 0.1% DCl (0.2 mM peptide). The peptides were treated by vortex (1 min) and sonication (5 min) to give a homogenous and transparent solution. An aliquot (70 µL) was placed on 25 mm × 4 mm CaF$_2$ plates (International Crystal Labs). Spectra were obtained using the Happ-Genzel method from 1550 to 1750 cm$^{-1}$ with a 2 cm$^{-1}$ resolution (512 scans).

4.6.5 Negative-stain TEM and electron diffraction

Fibril morphologies of the assembled peptide materials were characterized by TEM analysis. An aliquot of each assembled peptide (10 µL) was applied to 200-mesh carbon-coated copper grids. After standing for 30 s, excess fluid was removed by capillary action. Peptide fibrils were then stained by applying 10 µL of 5% uranyl acetate to the grid for 2 min; excess fluid was removed by capillary action. The peptides were washed with water for 1 s (10 µL) then the remaining solvent was removed by capillary action and the grids were allowed to dry prior to imaging. Electron micrograph images were obtained using a Hitachi 7650 transmission electron microscope in high-contrast mode at an accelerating voltage of 80 kV. Fibril dimensions were measured in ImageJ (http://rsbweb.nih.gov/ij/). The reported dimensions are the average of at least 100 measurements on distinct fibrils for each self-assembled peptide.
4.7 References


4.8 APPENDIX: Supporting Information and Data.

Figure 4.8.S1. Analytical HPLC Trace (215nm) for Ac-(FKFE)2-NH2.

Figure 4.8.S2. Analytical HPLC Trace (215nm) for Ac-(HphKHphE)2-NH2.

Figure 4.8.S3. Analytical HPLC Trace (215nm) for Ac-(WKWE)2-NH2.
Figure 4.8.S4. Analytical HPLC Trace (215nm) for Ac-(1-NalK1-NalE)₂-NH₂.

Figure 4.8.S5. Analytical HPLC Trace (215nm) for Ac-(2-NalK2-NalE)₂-NH₂.

Figure 4.8.S6. Analytical HPLC Trace (215nm) for Ac-(BipKBipE)₂-NH₂.
Figure 4.8.S7. Analytical HPLC Trace (215nm) for Ac-WKFEFKFE-NH$_2$.

Figure 4.8.S8. Analytical HPLC Trace (215nm) for Ac-(WKFE)$_2$-NH$_2$.

Figure 4.8.S9. Analytical HPLC Trace (215nm) for Ac-BipKFEFKFE-NH$_2$.
Figure 4.8.S10. Analytical HPLC Trace (215nm) for Ac-(BipKFE)$_2$-NH$_2$.

Figure 4.8.S11. MALDI-TOF-MS for Ac-(FKFE)$_2$-NH$_2$. 
Figure 4.8.S12. MALDI-TOF-MS for Ac-(HphKHphE)$_2$-NH$_2$.

Figure 4.8.S13. MALDI-TOF-MS data for Ac-(WKWE)$_2$-NH$_2$. 
Figure 4.8.S14. MALDI-TOF-MS data for Ac-(1-NalK1-NalE)_2-NH₂.

Figure 4.8.S15. MALDI-TOF-MS data for Ac-(2-NalK2-NalE)_2-NH₂.
Figure 4.8.S16. MALDI-TOF-MS data for Ac-(BipKBipE)$_2$-NH$_2$.

Figure 4.8.S17. MALDI-TOF-MS data for Ac-WKFEFKFE-NH$_2$. 
Figure 4.8.S18. MALDI-TOF-MS data for Ac-(WKFE)$_2$-NH$_2$.

Figure 4.8.S19. MALDI-TOF-MS data for Ac-BipKFEFKFE-NH$_2$. 
Figure 4.8.S20. MALDI-TOF-MS data for Ac-(BipKFE)_2-NH₂.

Table 4.8.S1. Calculate and Observed m/z for synthetic peptides (MALDI-TOF-MS).

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<th>[M+K]^+</th>
<th>[M+H]^+</th>
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Figure 4.8.S21. HPLC calibration curve (215nm) for Ac-(HphKHphE)$_2$-NH$_2$.

Figure 4.8.S22. HPLC calibration curve (215nm) for Ac-(WKWE)$_2$-NH$_2$. 
Figure 4.8.S23. HPLC calibration curve (215nm) for Ac-(1-NalK1-NalE)_{2}-NH_{2}.

Figure 4.8.S24. HPLC calibration curve (215nm) for Ac-(BipKBipE)_{2}-NH_{2}.
**Figure 4.8.S25.** HPLC calibration curve (215nm) for Ac-WKFEFKFE-NH$_2$.

**Figure 4.8.S26.** HPLC calibration curve (215nm) for Ac-(WKFE)$_2$-NH$_2$. 

![Graph](image-url)
Figure 4.8.S27. HPLC calibration curve (215nm) for Ac-BipKFEKFE-NH$_2$.

Figure 4.8.S28. HPLC calibration curve (215nm) for Ac-(BipKFE)$_2$-NH$_2$. 
Chapter 5

5 Conclusion and Future Work

5.1 Summary of Projects

Peptide self-assembly into β-sheet fibrils is of critical importance in amyloid pathology and has been exploited in the design of functional biomaterials. Amphipathic peptides composed of alternating hydrophobic and hydrophilic amino acids are a privileged class of peptide that have a high propensity to self-assemble into β-sheet fibrils. The Ac-(FKFE)_2-NH₂ peptide is an amphipathic sequence that has been extensively studied and forms putative β-sheet bilayer fibrils in which the hydrophobic
Phe side chains are organized to a single face of each constituent sheet; upon bilayer formation these hydrophobic benzyl groups are sequestered in the hydrophobic core of the resulting fibril. Herein we report explorations of peptide length, sequence order, and aromatic amino acid surface area on self-assembly of Ac-(FKFE)$_2$-NH$_2$-derived peptides.

5.1.1 Sequence Length Determinants for Ac-(FKFE)$_2$-NH$_2$.

Sequence length for amphipathic $\beta$-sheet peptides is one parameter that affects both self-assembly propensity and the emergent properties of the assembled putative $\beta$-sheet bilayer materials.$^{17-21}$ Work by Zhang et al where the repeating units of Ac-(FKFE)$_n$-NH$_2$ (where $n = 2$, 3, and 4) were modified presented data indicating that when $n = 2$ the kinetics were attenuated and the resulting biomaterials were not as robust, when compared to $n = 3$. Sch€n€ider et al showed that truncation of (VK)$_n$-NH$_2$ peptides by a single amino acid, resulting in an odd number of amino acids, failed to self-assemble.$^{27}$ These studies inspired us to investigate the effects of length for Ac-(FKFE)$_2$-NH$_2$ by truncating at the N- and C-terminus to form the odd-numbered derivatives: Ac-KFEFKFE-NH$_2$ and Ac-FKFKEFKF-NH$_2$.

We found that changes in peptide length had dramatic effects on the self-assembly of each peptide. Ac-FKFKEFKF-NH$_2$ was capable of self-assembling into fibrils with a unique morphology relative Ac-(FKFE)$_2$-NH$_2$ (Figure 5.1); however, the critical concentration required to induce assembly was slightly higher than the untruncated peptide, indicating that fibril formation is slightly less thermodynamically favorable. Ac-KFEFKFE-NH$_2$ peptide had a significantly reduced propensity to self-assemble at acidic pH but addition of pH 7 phosphate buffer promoted the formation of fibrils similar to the fibrils formed from Ac-FKFKEFKF-NH$_2$. We hypothesize that a change in strand registry
from out-of-register for even-numbered peptides to in-register for odd-numbered peptides explains these observed differences, although, further experimentation is required to determine the registry and full characterization of the $\beta$-sheets. Our findings highlighted the significance of sequence length on the self-assembly of short amphipathic peptides that have potential to be fine-tuned by minute changes to peptide length.

**Figure 5.1.** Effects of peptide length by a single amino acid on strand registry and fibril morphology for Ac-(FKFE)$_2$-NH$_2$.

### 5.1.2 Varied Sequence Pattern for Ac-(FKFE)$_2$-NH$_2$.

As discussed above, amphipathic peptides are composed of polar and nonpolar amino acid residues. The alternating hydrophobic and hydrophilic residues in Ac-(FKFE)$_2$-NH$_2$ form putative $\beta$-sheet bilayer which results in solvent exposed hydrophilic residues creating a soluble fibril. We designed four derivatives of Ac-(FKFE)$_2$-NH$_2$ to determine
whether fibrils could still form if the construction of the bilayer was perturbed by changing the sequence patterning.

Altering the location of the residues compared to the parent peptide resulted in numerous differences in the adoption of secondary structure and the formation of fibrils. The characteristic pattern of alternating hydrophobic and hydrophilic residues of the parent Ac-(FKFE)$_2$-NH$_2$ was maintained for Ac-(FK)$_2$(FE)$_2$-NH$_2$ (Figure 5.2). The remaining derivatives, however, are constructed with hydrophobic residues on both faces of the $\beta$-strand. It was determined that peptides with the where hydrophobic residues were present on both sides of the $\beta$-strand could still form $\beta$-sheet fibrils but at the cost of decreased thermodynamic fibril stability, as determined by a sedimentation assay which determined the critical concentration for peptide self-assembly, and changes to fibril morphology. These observations conclude that peptides with alternating hydrophobic and hydrophilic amino acids increase the propensity to form $\beta$-sheet fibrils and slight changes to the sequence order significantly impacts the resulting assembled materials.
Figure 5.2. Characteristic amphipathic β-sheet sequence pattern with alternating hydrophobic and hydrophilic residues compared to unconventional amphipathic sequence patterns.

5.1.3 Increased Surface Area of Amino Acid Side Chains within the Hydrophobic Core.

Amphipathic peptides with alternating hydrophobic and hydrophilic residues desolvate the hydrophobic side chains within a β-sheet bilayer.\textsuperscript{15,28,29} We previously reported that aromatic side chains are not required, but rather a certain degree of hydrophobicity is necessary to induce self-assembly. Aromatic side chains have a higher degree of hydrophobicity, thus, increasing the propensity to self-assemble. To investigate
the capacity for increased surface area within amphipathic $\beta$-sheet bilayer fibrils, we probed the effects of increasing the surface area of aromatic side chains using natural and nonnatural amino acids in the sequence Ac-(XKXE)$_2$-NH$_2$ (where X = phenylalanine (Phe), homophenylalanine (Hph), tryptophan (Trp), 1-naphthylalanine (1-Nal), 2-naphthylalanine (2-Nal), or biphenylalanine (Bip)).

Substitutions at X with various residues caused formation of $\beta$-sheet fibrils, each with differing properties depending on the residue at the X position. Interestingly, increasing the surface volume resulted in narrower fibril widths with varying morphologies for most substitutions used (Figure 5.3). This correlation between increased side chain surface area and decreased fibril widths is not understood and provides additional aspects to explore in future research efforts. These data expand the common motifs used in peptide design of peptides with the sequence Ac-(XKXE)$_2$-NH$_2$ and similar peptides by suggesting that large molecular volume is accepted within the hydrophobic core of $\beta$-sheet bilayer fibrils and can be used to develop novel biomaterials containing nonnatural amino acids that could provide higher tolerance to proteolytic degradation.
Figure 5.3. Increased surface area of amino acid side chains within the hydrophobic bilayer for Ac-(XKXE)$_2$-NH$_2$ β-sheet bilayer fibrils.
5.2 Future Efforts

It is hypothesized that amphipathic peptides with alternating hydrophobic and hydrophilic residues assemble into fibrils with a putative $\beta$-sheet bilayer. Typically, these peptides are soluble in aqueous media because the hydrophobic residues are buried within a hydrophobic bilayer that is surrounded by a hydrophilic shell. Previous research has shown that materials like these are capable of forming hydrogels when the peptide is at high enough concentrations.\(^{22-24}\) At a high concentration, the fibril network becomes so dense that water molecules are encapsulated reducing the flow of free water and causing the formation of a gelatinous environment. Peptide hydrogels can provide a biocompatible environment for many biomedical applications such as cell scaffolding,\(^{30-33}\) tissue engineering,\(^{34-37}\) and drug delivery vectors,\(^{38-46}\) Despite the large body of literature on biomedical applications of peptide self-assembly, most reports are focused on application of these materials, rather than on structural determination and the effects necessary to induce and tune their functional properties via structure. Additional studies are required in this area in order to understand the relationship of self-assembly of amphipathic peptides and the emergent properties of the resulting materials.

5.2.1 High-resolution structural studies to confirm bilayer architectures.

The hypothesis that $\beta$-sheet peptides form a bilayer is based on inference from low-resolution techniques. High-resolution structural studies of these types of materials is challenging.\(^{47}\) High-resolution X-ray crystallography has not been successful in determining exact architecture because amphipathic bilayer fibrillar peptides tend to form large soluble fibrils that do not crystallize. Advances are slowly being made using solid-state NMR and isotopic-edited FT-IR (IE FT-IR) to determine strand registry; however,
these techniques have not yet been widely adopted. (91) Although circular dichroism and X-ray diffraction patterns in conjunction with TEM imaging provide details into the secondary structure and fibril morphology, they are not as conclusive as a crystallographic structure, or structures determined by solid-state NMR experiments. Therefore, future endeavors should focus on validating the proposed β-sheet bilayer that we and others have hypothesized.

5.2.2 **Distinguishing lamination patterns and thermodynamic stability.**

Peptide β-sheets do not infinitely laminate into large fibrils with undefined widths and thicknesses but rather retain a specific fibril width and morphology via a thermodynamic equilibrium. This equilibrium is reached through two proposed lamination patterns; edge-to-edge and face-to-face (Figure 5.4). In face-to-face packing, the hydrophilic residues “stack,” leaving the terminal hydrophobic residue solvent exposed. In edge-to-edge packing, the peptides meet and laminate at the exposed termini and extend until they reach a terminal length (equilibrium) and association does not increase at any appreciable rate. It is currently not fully understood which pattern exists, if any, within fibrils of this type. The driving forces for specific lamination modes is also not understood. Efforts to understand these lamination modes are necessary in order to fully be able to design precisely tuned materials derived from these types of peptides.
5.2.3 Defining strand registry using novel synthetic peptides.

It has been proposed that peptides having alternating hydrophobic and hydrophilic amino acids with an odd or even number can be in-register or out-of-register, respectively. Designing an amphipathic peptide with an odd of residues and complementary electrostatic interactions with a propensity to form β-sheet bilayer fibrils could enhance the emergent properties of self-assembly. Schneider et al proposed that odd-numbered \((VK)_nV-NH_2\) peptides assembled because the peptide monomer were in-register with hydrophobic valine side chains at the \(N\)- and \(C\)-terminus. Further investigation of this hypothesis for odd-numbered peptides could be conducted using the \(Ac-(XKXE)_nZ(KXEX)_n-NH_2\) and \(Ac-(KXEX)_nZ(XKXE)_n-NH_2\) peptides (where \(X\) is a
hydrophobic residue and Z is any amino acid) because they are in-register with the same amino acid composition but the flanking residues at the N- and C-terminus are either hydrophobic or hydrophilic, respectively. Using this data, we can either validate or disprove our hypothesis in the context of a different, yet similar system.

Various other substitutions in the peptide sequences Ac-((XKXE)_nZ(KXEX)_n)-NH₂ and Ac-((KXEX)_nZ(KXXE)_n)-NH₂ could be used in the creation of novel biomaterials. Incorporation of Cys in the center could also be used to promote cross-linking for fibril stability, similar to tyrosine cross-linking seen in PICUP experiments, which have also been used to stabilize the resulting structures formed. Also, cyclization of the peptide strand using Cys at the N- and C-termini can inhibit self-assembly until addition of reductive triggers to form the linear β-strand. Therefore, designing peptides to be in-register with complementary electrostatic interactions to promote self-assembly and incorporation of Cys to regulate self-assembly may have the potential to become novel biomaterials with enhanced emergent properties.

5.2.4 Comparison of hydrogel rigidities with varying fibril morphologies.

Studies should be conducted to investigate the correlation of fibril morphology to hydrogel rigidity. Many of our peptides had unique fibril morphologies despite their similar amino acid compositions. It would be interesting to determine if the changes in morphology including helical pitch, thickness, bundling, and mixed morphologies as visualized by TEM influence the hydrogel properties of these peptides. (Figure 5.5). This, of course, is merely conjecture because it is unknown if these systems are capable of forming gels, so initial conditions to promote gelation needs to be established. Because each of these peptides form such drastically different fibrils, but yet the sequences are so
similar, it provides an opportunity to probe if fibril morphology influences hydrogelation properties.

**Figure 5.5.** Comparison of fibril morphologies for peptides with similar amino acid compositions.
5.3 Summary

Peptide self-assembly is a naturally occurring phenomenon that can be exploited to form elegant functional structures as seen in spider silk and collagen, but also capable of leading to the deleterious plaques seen in Alzheimer’s disease patients and causing malfunctions in pancreas functions like in Type II diabetes patients. If scientists can learn to control and manipulate the interactions that govern the assembly of peptides, it will allow for the creation of new types of materials that are biocompatible and capable of initiating a biological function that can be triggered by an environmental stimulus.

Further, in learning to control and create these materials, it should also provide insight into unregulated, detrimental amyloid formation and assist in developing ways to abrogate or perturb unregulated peptide self-assembly. Therefore, this thesis focused on determining the effects of minute change to the peptide sequence and the subsequent impact these alterations had on the self-assembly process and resulting assembled materials for the self-assembling amphipathic Ac-(FKFE)$_2$-NH$_2$ peptide.

It was found that peptide length, sequence order, and aromatic amino acid substitution exert a dramatic influence on self-assembly propensity, fibril morphology, and thermodynamic stability of the resulting materials. In terms of peptide length, truncated derivatives at the $N$- or $C$-terminus had significantly different self-assembling propensity and fibril morphology. The $C$-terminus truncated derivative, Ac-FKFEFKF-NH$_2$, assembled into fibrils with unique morphology relative to the parent peptide. However, the $N$-terminus truncated, Ac-KFEFKFE-NH$_2$, had a strongly reduced propensity to self-assemble, even failing to self-assemble altogether at acidic pH. Similar discoveries were observed when the sequence order was changed from alternating
hydrophobic/hydrophilic amino acids to sequence patterns less likely to form a hydrophobic β-sheet bilayer fibrils. The Ac-KEFFFFKE-NH$_2$ peptide showed a dependency on peptide concentration while the Ac-(KFFE)$_2$-NH$_2$ and Ac-FFKEKEFF-NH$_2$ both failed to form higher ordered fibril-like structures at acidic pH. However, under neutral conditions self-assembly was induced for all derivatives except Ac-(KFFE)$_2$-NH$_2$ remained unassembled. Lastly, increased aromaticity and hydrophobicity of amphipathic Ac-(XKXE)$_2$-NH$_2$ peptides proved that self-assembly can accommodate sterically bulky functional groups within the hydrophobic bilayer. However, co-solvent is required to solubilize the more hydrophobic nonnatural amino acids. These results indicate that peptide length, amino acid sequence patterns, and aromatic substitution dramatically impact peptide self-assembly and the resulting assembled materials. However, theses minute changes to peptide sequence can be exploited as a variable in the creation of novel materials composed of self-assembled peptides.
5.4 References


