Assembly and Coassembly of Peptides Derived from β-Sheet Regions of β-Amyloid

Dissertation

submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Chemistry

by

Nicholas L. Truex

Dissertation Committee:
Professor James S. Nowick, Chair
Professor Greg Weiss
Professor Robert Spitale
DEDICATION

In memory of my mother,

Michelle L. Truex
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CURRICULUM VITAE

Department of Chemistry  
University of California, Irvine  
1102 Natural Sciences II  
Irvine, CA 92697-2025

E-mail: nltruex@gmail.com  
Tel: (317) 372-5786

EDUCATION

Ph.D., Chemistry, University of California, Irvine  2012 – 2017
B.S., Chemistry, Purdue University  2007 – 2011

RESEARCH EXPERIENCE

Graduate Student with Professor James S. Nowick, U.C. Irvine  2012 – 2017
Undergraduate Researcher with Professor Ei-ichi Negishi, Purdue  2008 – 2012
Summer Intern, Eli Lilly and Company  2008 – 2010

PUBLICATIONS (an asterisk * indicates the corresponding author)


CONFERENCE PRESENTATIONS (an underline indicates the presenter)

6. “Assembly and Coassembly of Peptides Derived from β-Sheet Regions of β-Amyloid”
   Truex, N.L.; Wang, Y.; Nowick, J.S. Presented at the 2016 Graduate Research Symposium

5. “Effect of Hydrophobicity and Charge in the Oligomerization of Amyloidogenic Peptides
   and the Design of a pH-Switchable Oligomer” Wang, Y.; Truex, N.L.; Wali, H.A.M.;
   Nowick, J.S. Presented at the 45th Western Regional Meeting of the American Chemical
   Society, San Marcos, CA, November 6–8, 2015.

4. “A β-Hairpin Derived from Transthyretin 106–121 that Forms Square Hydrophobic Channels”
   Yoo, S.; Truex, N.L.; Kreutzer, A.G.; Nowick, J.S. Presented at the 45th Western Regional
   Meeting of the American Chemical Society, San Marcos, CA, November 6–8, 2015.

3. “Mimicry and Study of Amyloidogenic Peptides with Macrocyclic β-Sheet Models” Ferrick,
   K.R.; Truex, N.L.; Nowick, J.S. Presented at the 44th National Organic Chemistry

2. “Assembly of Peptides Derived from the Central and C-Terminal Regions of β-Amyloid”
   Truex, N.L.; Nowick, J.S. Presented at the 2015 Data Science Initiative Summer Fellows

   Wang, Y.; Dong, V.; Nowick, J.S. 2013. Presented at the 246th National Meeting of the
   American Chemical Society, Indianapolis, IN, September 8–12, 2013.

EXPERIMENTAL SKILLS
Spectrometry, HPLC Chromatography, Circular Dichroism (CD), Infrared Spectroscopy
(IR), UV-Vis Spectroscopy, Dynamic Light Scattering (DLS), SDS-PAGE, Analytical
Ultracentrifugation.

TEACHING EXPERIENCE
Teaching Assistant, Department of Chemistry, University of California, Irvine
Chem 1C, General Chemistry 2017
Chem 51B, Organic Chemistry 2015
Chem 203, Organic Spectroscopy (Graduate Course) 2014, 2015
Chem 1LD, Organic Chemistry Laboratory 2014
Chem 51LB, Organic Chemistry Laboratory 2014
Chem 52LA, Honors Organic Chemistry Laboratory 2013
Chem 193, CalTeach Research Methods 2013
Chem 1C, General Chemistry 2012, 2013

Get FIT! Faculty in Training Program with Associate Professor Suzanne A. Blum
Chem 203, Organic Spectroscopy (Graduate Course) 2014
HONORS AND AWARDS
Allergan Graduate Fellowship 2016
Michael Zach Award 2016 – 2017
   In recognition of the most promising future faculty member at UC Irvine on the basis of research and teaching.
Regents’ Dissertation Fellowship Award 2017

DEPARTMENT CONTRIBUTIONS
NMR Facility Fellow 2016

PROFESSIONAL AFFILIATIONS
American Chemical Society, member 2013 – 2017
ABSTRACT OF THE DISSERTATION

Assembly and Coassembly of Peptides Derived from β-Sheet Regions of β-Amyloid

By
Nicholas L. Truex

Doctor of Philosophy in Chemistry
University of California, Irvine, 2017

Professor James S. Nowick, Chair

Interactions among β-sheets are critical in driving the aggregation of the β-amyloid peptide (Aβ) to form oligomers and fibrils in Alzheimer’s disease. Two main regions of this 40- or 42-residue peptide are known to adopt β-sheet structure and promote aggregation: the central region and the C-terminal region. The central region comprises the hydrophobic pentapeptide LVFFA (Aβ17–23), and the C-terminal region comprises the hydrophobic undecapeptide AIIGLMVGGVV (Aβ30–40) or the hydrophobic tridecapeptide AIIGLMVGGVVIA (Aβ30–42). This dissertation is devoted to studies of the assembly and coassembly of peptides derived from the central and C-terminal regions of Aβ, which are critical in the formation of oligomers and fibrils in Alzheimer’s disease pathology.

Chapters 2 and 3 of this dissertation describe a two-part investigation of peptides derived from the central and C-terminal regions of Aβ. The first part, called “Assembly of Peptides Derived from β-Sheet Regions of β- Amyloid”, introduces two macrocyclic β-sheet peptides that contain residues 17–23 (LVFFAED) from the central region and residues 30–36 from the C-terminal region, and elucidates how each peptide self-assembles in aqueous solution. 1H NMR spectroscopy shows that the peptides assemble to form tetramers. Incorporation of a single isotopic label into each peptide and 15N-edited NMR spectroscopy facilitated the identification and quantification of the monomers
and tetramers. Molecular modeling further elucidates the structures of the tetramers. These studies provide insights into the peptide interactions and supramolecular assembly of an important peptide in an important amyloid disease.

The second part, called “Coassembly of Peptides Derived from β-Sheet Regions of β-Amyloid”, asks how the macrocyclic β-sheet peptides coassemble upon mixing. This question is important because the two regions generally segregate in most fibril structures of Aβ but coassemble in the oligomers. The two macrocyclic β-sheet peptides form a complex mixture homotetramers and heterotetramers upon mixing. 15N-Edited NMR spectroscopy shows that three heterotetramers form in addition to the homotetramers. Job’s method of continuous variation and nonlinear least-squares fitting to help establish the identity of each heterotetramer, and also reveal a surprising preference for one particular heterotetramer. These studies illustrate the role of molecular recognition in amyloid assembly and push the boundaries for experimental studies involving complex supramolecular equilibria.

For the remainder of this dissertation, the focus of the research shifts to a study on macrocyclic β-sheets that contain N-methyl blocking groups, rather than Hao. These studies were intended to conclude ongoing efforts to characterize analogues of peptides derived from Aβ17–36. These analogues were studied by X-ray crystallography and 1H NMR spectroscopy, and also with biophysical and biological techniques. DOSY NMR studies revealed these peptides oligomerize in solution, which have not been observed previously by other biophysical techniques. Additional NMR experiments, and corroboratory analytical ultracentrifugation studies, are needed to further establish this finding. These studies show that evaluating the oligomers N-methyl peptides is possible by 1H NMR spectroscopy. These efforts to correlate solution-phase biophysical techniques with 1H NMR spectroscopy may prove useful to other members the Nowick lab.
CHAPTER 1

An Overview of using Chemical Model Systems to Study

Amyloid Aggregation
INTRODUCTION

In this chapter, I attempt to give a literature summary of research that has helped inspire this dissertation. Although the following chapters contain their own research summaries, the current chapter provides wider context. The topics discussed in this chapter are divided into five sections. The first section gives a broad overview of peptide and protein aggregation in amyloid diseases, which is followed by a similar section that focuses primarily on the aggregation of the \( \beta \)-amyloid peptide (A\( \beta \)) in the pathology of Alzheimer’s disease. The third section introduces chemical model systems derived from amyloidogenic peptides and proteins, and explains advantages in studying these model systems. The fourth section introduces the chemical model systems that are studied in the following chapters of this dissertation, which are called macrocyclic \( \beta \)-sheet peptides. The concluding section describes several discoveries that I made during the first year of my graduate studies at UC Irvine and provides context for the studies described in the following chapters of this dissertation.

1. PEPTIDE AND PROTEIN AGGREGATION IN AMYLOID DISEASES

Amyloid aggregation is the unifying theme of all amyloidogenic diseases, and is emerging as an important part of the protein energy landscape.\(^1,2,3\) Over 60 peptides or proteins have been identified thus far that can aggregate to form amyloid oligomers and fibrils. Many of these amyloids are important in the pathology of amyloid diseases, such as \( \beta \)-amyloid (A\( \beta \)) in Alzheimer’s disease, \( \alpha \)-synuclein in Parkinson’s disease, and hIAPP in type II diabetes.\(^1,3\) Other amyloids appear to have vital biological roles. A growing body of work suggests that amyloids are important in protein storage, cell-to-cell communication, and biofilm formation.\(^1,4\) As additional new amyloids are identified and characterized, other important properties will undoubtedly be discovered.
Characterizing the structures of amyloid oligomers and fibrils is the bedrock for elucidating their roles in biology and disease. The structures of many amyloid fibrils have been established by solid-state NMR spectroscopy, and more recently by cryo-electron microscopy, for several full-length amyloidogenic peptides or proteins, including \( \text{A}\beta_{1-40} \), \( \text{A}\beta_{1-42} \), hIAPP, \( \alpha \)-synuclein, and Tau. These fibrils are typically composed of parallel \( \beta \)-sheets that assemble in an elongated fashion, and then twist together. Although amyloid fibrils are generally nontoxic aggregates, fibril formation usually results in the loss of function of the peptide or protein involved.

Amyloid oligomers formed by most peptides and proteins are damaging to cells, regardless of whether the corresponding protein is associated with the pathology of a known disease. Oligomerization results in a heterogeneous mixture of intermediates that are difficult to characterize by high-resolution techniques, such as NMR spectroscopy or X-ray crystallography. Most efforts to characterize the oligomers have focused on evaluating their size and secondary structure, which have offered little information on their high-resolution structures. At this time, a high-resolution structure has not been determined for any oligomer of a full-length amyloidogenic peptide or protein. As a result, little is known about the precise roles of the oligomers in amyloid diseases.

2. A\( \beta \) AGGREGATION IN ALZHEIMER'S DISEASE

A\( \beta \) in the pathology of Alzheimer’s disease is generated as a 40- or 42- residue peptide that aggregates to form amyloid oligomers and fibrils. Even though the fibrils have been associated with the progression of the disease pathology for over a century, the oligomers have emerged over the last two decades as the primary species that promote neurodegeneration. The
characterization of Aβ oligomers and fibrils represents the forefront of understanding the mechanisms by which they form and contribute to Alzheimer’s disease pathology.

Aβ oligomers adopt a heterogeneous mixture of aggregates that are difficult to characterize and study.\textsuperscript{16,17} Mixtures of Aβ oligomers composed of dimers, trimers, tetramers, hexamers, and even dodecamers have been observed by gel electrophoresis,\textsuperscript{18} atomic force microscopy,\textsuperscript{19} fluorescence lifetime imaging,\textsuperscript{20} ion mobility mass spectrometry,\textsuperscript{21} and by other methods. Information on the secondary structure of these oligomers has been obtained by making use of circular dichroism spectroscopy,\textsuperscript{22} electron paramagnetic resonance,\textsuperscript{23} two-dimensional infrared spectroscopy,\textsuperscript{24} conformation-specific antibodies,\textsuperscript{22,25} and affibody proteins.\textsuperscript{26,27} These biophysical techniques show that Aβ adopts antiparallel β-sheets within the oligomers, and may even favor a β-hairpin conformation. Figure 1.1 shows a β-hairpin conformation of Aβ\textsubscript{1–40} that has been isolated and stabilized by an affibody protein.

![Figure 1.1. β-Hairpin conformation of Aβ\textsubscript{1–40} (green) with an affibody protein (PDB ID: 2OTK).](image)

Further aggregation of Aβ results in the formation of amyloid fibrils. The structures of these fibrils are important, because they reveal key regions of the peptide that can adopt β-sheet
These β-sheet-structured regions in Aβ fibrils may also favor β-sheets in Aβ oligomers. The fibril structures of Aβ₁–₄₀ typically favor extended parallel β-sheet structures in which the central region and C-terminal regions laminate together, with a U-shaped turn in between (Figure 1.2A). This fibril structure of Aβ₁–₄₀ is discussed in more detail in Chapter 2. The structures of Aβ₁–₄₂ fibrils also form parallel β-sheets, but these structures tend to be more compact (Figure 1.2B).

Figure 1.2. Solid-state NMR structures of fibrils formed by Aβ₁–₄₀ (A, PDB ID: 2LMQ) and Aβ₁–₄₂ (B, PDB ID: 2NAO).

3. CHEMICAL MODEL SYSTEMS

The heterogeneity and polymorphism of amyloid oligomers, and even some fibrils, continues to limit their isolation and characterization to this day. These challenges have led to designs and studies of chemical model systems that contain smaller amyloidogenic fragments. These chemical model systems are peptide scaffolds composed of one or two peptide strands, which contain key amyloidogenic regions from a peptide or protein. These regions are often identified as those that favor β-sheet structure in the fibrils. These regions may also be identified through evaluating the aggregation propensity of peptide sequences by either experimental or computational techniques. Incorporation of these amyloidogenic
regions into a chemical model system facilitates the characterization and study of these regions within a well-behaved system.

The advantage to studying these chemical model systems is the information they can provide: high-resolution structures of amyloid-like oligomers and fibrils. X-ray crystallography has revealed the structures of amyloid-like oligomers and fibrils of chemical model systems derived from Aβ,30,36,37,38 Tau,36 α-synuclein,39 hIAPP,40,41 transthyretin,42 αB-crystallin,43 β2-microglobulin,44 prion protein,45 and many other amyloidogenic peptides and proteins. 1H NMR spectroscopy studies have also provided information on the solution-phase assembly of chemical model systems derived from Aβ46,47,48,49,50 and hIAPP.41,51 These chemical model systems adopt structures of amyloid-like oligomers or fibrils that, in many cases, appear to resemble the oligomers or fibrils of their corresponding full-length peptide or protein.

Correlating structure with function is important for evaluating the biological significance of these chemical model systems. Solution-phase biophysical and biological studies have shown that some chemical model systems can mimic the biophysical and biological properties of their corresponding full-length peptide or protein, such as oligomerization and toxicity.37,43,52 These studies can also reveal themes and patterns associated with the molecular pathology of Alzheimer’s disease and other amyloid diseases.

4. MACROCYCLIC β-SHEET PEPTIDES

The Nowick laboratory has designed and introduced several chemical model systems to study the folding and oligomerization of β-sheets. These chemical model systems are called macrocyclic β-sheet peptides. The peptides contain turn and template units, which promote folding and prevent uncontrolled aggregation. Although the Nowick laboratory introduced these
peptides in 2007 as simple mimics of β-sheet folding and dimerization, these peptides have since been redesigned multiple times for studies of amyloid oligomers and fibrils.

In 2007, the Nowick laboratory introduced macrocyclic β-sheet peptides \( \text{I.} \) These peptides \( \text{I} \) contain a heptapeptide strand (upper strand) for displaying peptide fragments, two δ-linked ornithine (δOrn) units, and a template strand (lower strand) with two tripeptide mimics, called Hao (Figure 1.3A). The δOrn units connect the upper and lower strands, which permits β-sheet folding of these strands. The two Hao units promote the assembly of these peptides, but also block uncontrolled aggregation. Peptides \( \text{I} \) later inspired the Nowick laboratory to design and study the larger macrocyclic β-sheet peptides \( \text{2} \), which contain a nonapeptide strand in the upper strand (Figure 1.3B).

![Figure 1.3. Macrocyclic β-sheet peptides I, showing the heptapeptide strand in the upper strand and the two Hao units in the template strand (A). Macrocyclic β-sheet peptides 2, showing the nonapeptide strand and the two Hao units (B).](image)

\(^1\)H NMR and X-ray crystallography studies have shown that macrocyclic β-sheet peptides \( \text{I} \) and \( \text{2} \) can oligomerize to form dimers, tetramers, and other higher-order oligomers. In a study of an analogue of peptide \( \text{I} \) derived from the protein G variant NuG2, \(^1\)H NMR showed that this peptide forms hydrogen-bonded dimers that stack to form a sandwich-like tetramer. X-ray crystallography further established the structure of the hydrogen-bonded dimer. Figure 1.4A shows the crystal structure of the dimer of peptide \( \text{I} \). \(^1\)H NMR studies of an analogue of peptide \( \text{2} \) derived from A\( \beta_{15-23} \) showed that this peptide can also forms hydrogen-bonded dimers, which further...
assemble to form a tetramer.\textsuperscript{48} X-ray crystallography also showed a different tetramer morphology of peptide 2.\textsuperscript{57} Figure 1.4B shows the crystal structure of a cruciform tetramer formed by peptide 2.

![Figure 1.4](image)

**Figure 1.4.** X-ray crystallographic structure of a dimer formed by a macrocyclic β-sheet peptide 1 derived from the protein G variant NuG2 (A, PDB ID: 3NI3). X-ray crystallographic structure of a cruciform tetramer formed by a macrocyclic β-sheet peptide 2 derived from Aβ\textsubscript{15–23} (B, PDB ID: 4IVH).

While studying peptides 1 and 2, the Nowick laboratory worked on designing improved macrocyclic β-sheets that better mimic β-sheet folding and assembly. In 2012, the Nowick laboratory introduced macrocyclic β-sheet peptides 3 for delaying the aggregation of full-length Aβ (Figure 1.5A).\textsuperscript{60} Peptides 3 are similar to peptides 2, except that they contain only a single Hao in the template strand, rather than two. The four residues (R_8–R_{11}) that flank Hao add the ability to tune the hydrophobicity and charge of these peptides. The Nowick laboratory envisioned peptides 3 would exhibit enhanced folding and assembly over the predecessor peptides, 1 and 2.
Figure 1.5. Macrocyclic β-sheet peptides 3, showing the heptadopeptide strand and the single Hao unit in the template strand. X-ray crystallographic structure of a tetramer formed by a macrocyclic β-sheet peptide 3 derived from Aβ30–36 (B, PDB ID: 3T4G).

The Nowick laboratory characterized analogues of peptides 3 derived from Aβ, hIAPP, and other amyloidogenic peptides by X-ray crystallography and NMR spectroscopy. X-ray crystallography studies showed that an analogue derived from Aβ30-36 can assemble to form amyloid-like oligomers. These oligomers consist of hydrogen-bonded dimers that further assemble to form a barrel-shaped tetramer (Figure 1.5B).\textsuperscript{36,60} \textsuperscript{1}H NMR spectroscopy showed that peptides 3 exhibit enhanced β-sheet folding over the predecessor peptides 1 and 2. Although these studies of peptide 3 folding showed promise for using \textsuperscript{1}H NMR spectroscopy to characterize oligomers, the oligomers that formed were not well defined. As a result, hydrophilic residues were placed at position R8 and R11, which typically prevented oligomerization altogether.

In 2013, the Nowick laboratory began to develop macrocyclic β-sheets that do not contain any Hao units.\textsuperscript{61} These efforts led to the design of macrocyclic macrocyclic β-sheet peptides 4 (Figure 1.6).\textsuperscript{38} These peptides contain heptapeptide strands in the upper and lower
strands (R₁–R₇ and R₈–R₁₄). Peptides 4 also contain an N-methyl (N-Me) group, rather than Hao, to block uncontrolled aggregation.  

**Figure 1.6.** Macro cyclic β-sheet peptides 4, showing the two heptaptide strands (upper and lower strands) and the N-Me group.

In 2014, the Nowick lab published X-ray crystallographic structures of several analogues of peptides 4 derived from Aβ₁₇–₃₆. The crystal structures showed that these peptides can form triangular trimers, hexamers, and dodecamers (Figure 1.7). These oligomers assemblies inspired our laboratory to design several subsequent analogues derived from Aβ, β₂-microglobulin, α-synuclein, and transthyretin. X-ray crystallographic studies of these peptides show that they also assemble to form various sizes and morphologies of dimers, trimers, hexamers, dodecamers, and other higher-order oligomers. Solution-phase biophysical and biological studies of these peptides have also begun to show relationships between structure and toxicity of trimers and higher-order oligomers. These findings may reflect the structures and the toxicities of oligomers that full-length Aβ can form.

**Figure 1.7.** X-ray crystallographic structure of a trimer, hexamer, and dodecamer of formed by an analogue of macrocyclic β-sheet peptide 4 derived from Aβ₁₇–₃₆ (A-C, PDB ID: 4NTR).
5. MY DISSERTATION RESEARCH

In the summer of 2012 before starting my graduate studies at UC Irvine, I made two discoveries with macrocyclic β-sheet peptides 3 during a rotation in the Nowick laboratory. I found that macrocyclic β-sheet peptides 3 indeed form well-defined oligomers in solution, which had not been observed previously. I also found that two homologues of peptides 3, which were derived from Aβ17-23 (LVFFAED) and Aβ30-36 (AIIGLMV), can assemble and coassemble in solution. During the course of my graduate studies, I confirmed these findings by 1H NMR spectroscopy, which ultimately resulted in two first-author publications46,47 and are described in Chapters 2 and 3 of this dissertation. These findings also had a major influence on the publications41,51 and dissertation of my colleague, Yilin Wang.

For the remainder of my graduate career, I shifted the focus of my research to study peptides 4. These studies focus on characterizing analogues of peptides 4 derived from Aβ17–36. In Chapter 4, I describe my efforts to characterize these analogues by X-ray crystallography and 1H NMR spectroscopy, and also with biophysical and biological techniques.
REFERENCES


CHAPTER 2

Assembly of Peptides Derived from β-Sheet Regions of β-Amyloid
INTRODUCTION

Interaction among β-sheets is the two-edged sword in protein structure, imparting folding and stability but also driving misfolding and aggregation. While folding is typically associated with normal biological function, aggregation is associated with the pathology of Alzheimer's disease and other amyloid diseases, including Parkinson's disease and type II diabetes. In Alzheimer's disease, the β-amyloid peptide (Aβ) aggregates to form oligomers and fibrils that characterize the disease pathology.

Elucidation of the oligomers and fibrils is critical to understanding how Aβ aggregates and counteracting the harmful effects. The fibrils mark the thermodynamic end point of Aβ aggregation and accumulate as the disease progresses. Several high-resolution structures have been reported of the Aβ fibrils, which typically adopt parallel β-sheet structure. The oligomers are thought to be primarily responsible for neurodegeneration, causing synaptic dysfunction in neurons. The oligomers are metastable and heterogeneous, and thus are difficult to study by high-resolution structural techniques.

Two key regions of Aβ favor β-sheet formation and promote aggregation: the central region and the C-terminal region. The central region contains Aβ17–21 (LVFFA). The two phenylalanine residues therein are especially important in nucleating and propagating the formation of Aβ aggregates. The C-terminal region comprises residues AIIGLMVGGVV (for Aβ1–40) or AIIGLMVGGVVIA (for Aβ1–42). These successive hydrophobic residues also promote aggregation.

The central and C-terminal regions of Aβ are thought to assemble in a different fashion in the fibrils than in the oligomers. In fibrils formed by Aβ1–40, the two regions of the peptide can assemble to form layered parallel β-sheets connected by a U-shaped turn: one layer consists of
the central region and the other consists of the C-terminal region.\textsuperscript{4,5} Figure 2.1 illustrates a layered β-sheet structure formed by Aβ₁₋₄₀.\textsuperscript{4c} These layered fibril structures can further assemble in twos and threes to form fibrils that exhibit two-fold or three-fold symmetry. In the oligomers, the central and C-terminal regions are thought to coassemble in an antiparallel fashion to form β-hairpins, which assemble to form the oligomers.\textsuperscript{10} These regions may also promote the assembly of Aβ to form higher-order oligomers.

**Figure 2.1.** Layered β-sheet structure formed by Aβ₁₋₄₀ within β-amyloid fibrils (PDB ID: 2LMQ).

In 2012, our research group introduced macrocyclic β-sheet peptides 1 as a model system to investigate the assembly of amyloidogenic peptides and proteins (Figure 2.2).\textsuperscript{11} Peptides 1 consist of a heptapeptide strand (R₁₋₇), a template strand, and two turn units. The heptapeptide strand displays amyloidogenic peptide sequences. The template strand contains the unnatural amino acid Hao and four additional residues (R₈₋₁₁) that help promote β-sheet structure. Hao is a tripeptide mimic that templates β-sheet hydrogen bonding and blocks uncontrolled aggregation.\textsuperscript{12} The δ-linked ornithine (δOrn) turn units on each side connect the two strands and allow β-sheet folding.\textsuperscript{13} Our research group incorporated hydrophilic residues at positions R₈ and/or R₁₁ to minimize oligomerization.
In this two-part investigation, I incorporated residues from the central and C-terminal regions of Aβ into peptides 1 to ask whether these regions prefer to coassemble or to segregate.\textsuperscript{14} To promote the formation of well-defined oligomers, I incorporated hydrophobic residues into positions R\textsubscript{8} and R\textsubscript{11}. The first part—the current chapter—determines how the two peptides assemble in aqueous solution. The second part—the accompanying chapter—determines whether the two peptides exhibit a special preference to coassemble when mixed.\textsuperscript{14} This question is important because the two regions generally segregate in the fibrils but coassemble in the oligomers.

To facilitate these studies, I incorporated $^{15}$N isotopic labels into peptides 1. Peptides [\textsuperscript{15}$^N$]1 contain a single $^{15}$N isotopic label at the R\textsubscript{4} position in the center of the heptapeptide strand (Figure 2.2). These peptides are readily prepared from commercially available $^{15}$N-labeled amino acids using solid-phase peptide synthesis. The $^{15}$N isotopic label provides a simple and effective spectroscopic probe to monitor assembly and coassembly by $^1H,^{15}N$ NMR spectroscopy.
RESULTS AND DISCUSSION

Design of Peptides Derived from the Central and C-Terminal Regions of Aβ. I incorporated residues LVFFAED (Aβ17–23) and AIIGLMV (Aβ30–36) into peptides 1, to give peptides 1a and 1b. I designed the peptides with a distinct hydrophobic surface to promote assembly by incorporating isoleucine residues at positions R₈ and R₁₁ of the template strand. I also designed the peptides with a hydrophilic surface to promote solubility and prevent uncontrolled aggregation by incorporating lysine residues at positions R₉ and R₁₀ of the template strand.

1H NMR studies show that peptides 1a and 1b assemble to form sandwich-like tetramers in aqueous solution.¹⁶ The tetramers consist of two β-sheet dimers that stack like slices of bread. The dimers are stabilized by hydrogen-bonding interactions between the amide backbones of the heptapeptide strands; the tetramers are stabilized by hydrophobic interactions between the hydrophobic surfaces of the dimers. The following subsections describe the elucidation of the tetramers by NMR spectroscopy.

**DOSY Shows That Peptides 1a and 1b Form Tetramers.** Our laboratory has previously used DOSY NMR studies and corroboratory analytical ultracentrifugation (AUC)
experiments to establish that related macrocyclic β-sheet peptides form tetramers. DOSY NMR studies of peptides 1a and 1b show that these macrocyclic β-sheets also form tetramers (Table 2.1). The DOSY spectrum of peptide 1a at 0.15 mM shows two sets of resonances: one set from the monomer, with a diffusion coefficient of $20.4 \times 10^{-11}$ m$^2$/s; the other set from the tetramer, with a diffusion coefficient of $12.6 \times 10^{-11}$ m$^2$/s. At 8.0 mM, the spectrum shows only the latter set of resonances with a diffusion coefficient of $11.8 \times 10^{-11}$ m$^2$/s. The DOSY spectrum of peptide 1b at 1.0 mM shows resonances from the monomer, with a diffusion coefficient $19.4 \times 10^{-11}$ m$^2$/s, and the spectrum at 16.0 mM shows resonances from the tetramer, with a diffusion coefficient of $11.9 \times 10^{-11}$ m$^2$/s.

Table 2.1. Diffusion coefficients ($D$) of peptides 1a and 1b in D$_2$O at 298 K

<table>
<thead>
<tr>
<th></th>
<th>MW$_{monomer}$ (Da)</th>
<th>MW$_{tetramer}$ (Da)</th>
<th>conc. (mM)</th>
<th>$D$ ($10^{-11}$ m$^2$/s)</th>
<th>oligomer state</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>1767</td>
<td>7068</td>
<td>0.15</td>
<td>20.4 ± 1.7</td>
<td>monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.15</td>
<td>12.6 ± 1.6</td>
<td>tetramer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.0</td>
<td>11.8 ± 1.0</td>
<td>tetramer</td>
</tr>
<tr>
<td>1b</td>
<td>1643</td>
<td>6572</td>
<td>1.0</td>
<td>19.4 ± 1.7</td>
<td>monomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16.0</td>
<td>11.9 ± 1.1</td>
<td>tetramer</td>
</tr>
</tbody>
</table>

The ratio of diffusion coefficients of a tetramer and monomer is typically 0.6. DOSY studies show that the oligomers of peptides 1a and 1b have diffusion coefficients of about $12 \times 10^{-11}$ m$^2$/s and the monomers have diffusion coefficients of about $20 \times 10^{-11}$ m$^2$/s. The ratio of the diffusion coefficients (0.6) is consistent with a tetramer.

Elucidation of the Peptide 1a Tetramer. Peptide 1a forms a tetramer that consists of two β-sheet dimers. The $^1$H NMR spectrum of peptide 1a at 8 mM in D$_2$O at 298 K shows one predominant set of resonances (Figure 2.3a). These resonances are associated with the tetramer. The resonances are disperse and exhibit distinct spectral features that reflect well-defined β-sheet structure: Seven of the 11 α-protons appear downfield of 5 ppm. The methyl proton resonance of A$_{21}$ appears at 0.5 ppm. The aromatic proton resonances of F$_{19}$ appear upfield of 7 ppm (6.3 to
The $^1$H NMR spectrum of peptide 1a at 0.15 mM in D$_2$O at 298 K shows resonances associated with both the monomer and the tetramer (Figure S2.1). The resonances of the monomer lack the distinct spectral features of the tetramer.

**Figure 2.3.** $^1$H NMR spectra of (a) peptide 1a and (b) peptide 1b at 8.0 mM in D$_2$O at 600 MHz and 298 K.

The magnetic anisotropy of the diastereotopic $\delta$-proton resonances of the $\delta$Orn turn units reflects $\beta$-sheet folding in peptides 1 and related macrocyclic $\beta$-sheets.$^{10a,12}$ In a well-folded macrocyclic $\beta$-sheet, the diasterotopic pro-$S$ $\delta$-protons appear about 0.6 ppm downfield of the pro-$R$ $\delta$-protons. In the tetramer of peptide 1a, the pro-$S$ $\delta$-protons appear 0.63 and 0.74 ppm downfield of the pro-$R$ $\delta$-protons. In the monomer, the pro-$S$ $\delta$-protons of peptide 1a appear 0.30 and 0.39 ppm downfield of the pro-$R$ $\delta$-protons. The magnetic anisotropies of these proton resonances indicate that the monomer is moderately folded, while the tetramer is well folded.

The NOESY spectrum of peptide 1a shows strong NOEs associated with the $\beta$-sheet folding and assembly of the tetramer. The spectrum shows a network of five strong NOEs
associated with β-sheet folding: between the α-protons of V_{18} and K_{10}, the α-protons of E_{22} and K_{9}, the α-proton of F_{20} and the proton at the 6-position of the unnatural amino acid Hao (HaoH_{6}), and the α- and δ-protons of the δOrn turn units (Figure S2.2). The spectrum shows two additional NOEs associated with β-sheet dimerization, between the α-protons of L_{17} and D_{23} and between the α-protons of F_{19} and A_{21} (Figure S2.2a). Figure 2.4 illustrates the dimer of peptide 1a consistent with these interlayer NOEs.
**Figure 2.4.** Dimer and tetramer of peptide 1a. Hydrogen-bonded dimer subunit (upper). Blue arrows illustrate intramolecular and intermolecular NOEs observed in the NOESY spectrum. Sandwich-like tetramer consisting of two hydrogen-bonded dimers (lower). The blue arrow illustrates the interlayer NOEs observed in the NOESY spectrum. The tetramer exhibits four-fold symmetry and four $I_{11}^{-}$-Hao$_{OMe}$ interactions, even though only one arrow is shown.

The NOESY spectrum shows additional NOEs associated with the stacking of two dimers to form a sandwich-like tetramer. The spectrum shows a pattern of NOEs between the methoxy protons of Hao (Hao$_{OMe}$) and the side-chain protons of $I_{11}$, and additional NOEs between the protons at the 3- and 4-positions of Hao (HaoH$_3$ and HaoH$_4$) and the $\delta$-methyl protons of $I_{11}$.
(Figure S2.3). Figure 2.4 illustrates the stacking of the two dimers of peptide 1a consistent with these interlayer NOEs.

**Elucidation of the Peptide 1b Tetramer.** Peptide 1b forms a similar tetramer, which also consists of two β-sheet dimers. The tetramer is considerably less stable than that formed by peptide 1a and is in equilibrium with substantial amounts of monomer at millimolar concentrations (Figure S2.4). The $^1$H NMR spectrum of peptide 1b at 8.0 mM in D$_2$O at 298 K shows two sets of resonances. These resonances appear in a 3:2 ratio of intensities, with the predominant set associated with the tetramer and the smaller set associated with the monomer (Figure 2.3b). The resonances are broadened, reflecting chemical exchange between the tetramer and the monomer on a ca. hundred-millisecond time scale. The resonances associated with the tetramer exhibit several distinct spectral features that reflect well-defined β-sheet structure: Five of the 11 $\alpha$-proton resonances appear downfield of 5 ppm. The methyl proton resonances of L$_{34}$ are shifted upfield of 0.5 ppm (0.38 and 0.12 ppm). The pro-$S$ δ-proton resonances of the δOrn turn units appear 0.65 and 0.69 ppm downfield of the pro-$R$ δ-proton resonances.

The monomer of peptide 1b lacks these distinct spectral features. The pro-$S$ δ-proton resonances of the δOrn turn units appear 0.16 and 0.19 ppm downfield of the pro-$R$ δ-proton resonances. The magnetic anisotropies of these proton resonances indicate that the monomer is poorly folded. In contrast to peptide 1a, the monomer of peptide 1b predominates at low millimolar concentrations. At concentrations below 1 mM, the spectrum shows almost exclusively the monomer and virtually no tetramer.

The NOESY spectrum of peptide 1b shows strong NOEs associated with β-sheet folding and weaker NOEs associated with β-sheet assembly. The spectrum shows a network of five strong NOEs associated with β-sheet folding: between the $\alpha$-protons of I$_{31}$ and K$_{10}$, the $\alpha$-protons
of M$_{35}$ and K$_9$, the pro-$R$ $\alpha$-proton of G$_{33}$ and the H$_{aoH_6}$ proton, and the $\alpha$- and $\delta$-protons of the $\delta$-Orn turn units (Figure S2.5). The spectrum shows an additional NOE associated with $\beta$-sheet dimerization, between the $\alpha$-protons of I$_{32}$ and L$_{34}$ (Figure S2.5a). The spectrum does not show a well-defined NOE crosspeak between the $\alpha$-protons of A$_{30}$ and V$_{36}$. The absence of a well-defined crosspeak may reflect broadening of the resonances through chemical exchange with the monomer and overlap with an exchange crosspeak, or it may reflect a lack of close contact between the two protons. Figure 2.5 illustrates the $\beta$-sheet folding and dimerization of peptide 1b consistent with these NOEs.
Figure 2.5. Dimer and tetramer of peptide 1b. Hydrogen-bonded dimer subunit (upper). Red arrows illustrate intramolecular and intermolecular NOEs observed in the NOESY spectrum. Sandwich-like tetramer consisting of two hydrogen-bonded dimers (lower). The red arrow illustrates the interlayer NOEs observed in the NOESY spectrum. The tetramer exhibits four-fold symmetry and four I11−HaoOMe interactions, even though only one arrow is shown.

The NOESY spectrum shows additional NOEs associated with the stacking of two dimers to form a sandwich-like tetramer. Like peptide 1a, peptide 1b exhibits a pattern of NOEs between the Hao protons and the I11 side-chain protons, and an additional NOE between HaoH3 and the δ-methyl protons of I11 (Figure S2.6). Figure 2.5 illustrates the stacking of the two dimers of peptide 1b consistent with these interlayer NOEs.
\(^{1}H,^{15}N\) HSQC Studies of the Tetramers Formed by Peptides \([^{15}N]1a\) and \([^{15}N]1b\).

I studied \(^{15}N\)-labeled homologues of peptides \(1a\) and \(1b\) by \(^{1}H,^{15}N\) HSQC to identify and quantify the tetramers. \(^{1}H,^{15}N\) HSQC is a mainstay in NMR spectroscopy of proteins, but is also useful for peptides. \(^{15}N\)-Isotopic labeling and the dispersion provided by the \(f_1\) (\(^{15}N\)) dimension resolves mixtures of peptides far better than is possible by homonuclear techniques.

I prepared peptides \([^{15}N]1a\) and \([^{15}N]1b\), which each contain a single \(^{15}N\)-labeled amino acid in the center of the heptapeptide strand. Peptide \([^{15}N]1a\) contains an \(^{15}N\)-labeled phenylalanine; peptide \([^{15}N]1b\) contains an \(^{15}N\)-labeled glycine. The \(^{15}N\) isotopic label provides a spectroscopic probe for each species containing the \(^{15}N\)-labeled peptide.

![Diagram of macrocyclic β-sheet peptide \([^{15}N]1a\)](image1)

![Diagram of macrocyclic β-sheet peptide \([^{15}N]1b\)](image2)

The \(^{1}H,^{15}N\) HSQC spectrum of peptide \([^{15}N]1a\) in 9:1 H\(_2\)O/D\(_2\)O at 8.0 mM and 293 K shows two crosspeaks; the \(^{1}H,^{15}N\) HSQC spectrum of peptide \([^{15}N]1b\) also shows two crosspeaks (Figure 2.6). The spectrum of peptide \([^{15}N]1a\) shows a weak crosspeak associated with the monomer and a strong crosspeak associated with the tetramer; these crosspeaks are designated 1 and 2, respectively. The spectrum of peptide \([^{15}N]1b\) shows crosspeaks of comparable intensities associated with the monomer and tetramer; these crosspeaks are designated 3 and 4, respectively. Table 2.2 summarizes the chemical shifts of these crosspeaks.
Figure 2.6. $^1$H,$^{15}$N HSQC spectra of (a) peptide [15N]1a and (b) peptide [15N]1b at 8.0 mM in 9:1 H₂O/D₂O at 600 MHz and 293 K.

Table 2.2. Chemical shifts of peptides [15N]1a and [15N]1b

<table>
<thead>
<tr>
<th>crosspeak</th>
<th>$\delta_{F20}$</th>
<th>$\delta_{G33}$</th>
<th>species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.32</td>
<td>122.3</td>
<td>A monomer</td>
</tr>
<tr>
<td>2</td>
<td>8.56</td>
<td>121.3</td>
<td>A4 tetramer</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>8.39 112.5</td>
<td>B monomer</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>9.33 115.8</td>
<td>B$_4$ tetramer</td>
</tr>
</tbody>
</table>

$^a$1H,15N HSQC spectra were recorded at 8.0 mM in 9:1 H₂O/D₂O at 293 K.

In the accompanying chapter, I combine $^{15}$N-labeling and $^1$H,15N NMR spectroscopy to identify and characterize the seven different species that form upon mixing peptides [15N]1a and [15N]1b.$^{15}$
15N-Edited NOESY. I used peptides [15N]1a and [15N]1b to corroborate the pairing of the dimers within the tetramers. I recorded 1H, 15N NOESY-HSQC spectra with typical NOESY parameters in both 1H dimensions (f1 and f3), but with only one increment in the 15N dimension (f2). The result is an 15N-edited NOESY spectrum that shows only NOEs involving the 15NH protons and requires no more time than a regular NOESY spectrum.

The NH protons of an antiparallel β-sheet typically give a pattern of four key NOEs associated with β-sheet folding and interstrand interaction. Two of the NOEs reflect β-sheet folding: a weaker intraresidue NOE to the α-proton and a stronger interresidue NOE to the α-proton of the adjacent residue. Figure 2.7 illustrates these close contacts and shows typical distances (3.0 Å and 2.2 Å, respectively). Two of the NOEs reflect interstrand interaction: an NOE to the α-proton diagonally across in the non-hydrogen-bonded pair, and another NOE to the NH proton diagonally across in the hydrogen-bonded pair. Figure 2.7 also illustrates these close contacts and shows typical distances (3.2 Å and 3.3 Å, respectively). The magnitude of the interresidue NOE should be much stronger than the magnitude of the interstrand NOEs, because the NOE intensities decrease with distance to the inverse sixth power.

![Figure 2.7](image_url)

**Figure 2.7.** Four close contacts involving NH protons and Hα protons in antiparallel β-sheets. Typical distances are shown in angstroms.

The 15N-edited NOESY spectrum of peptide [15N]1a shows two sets of NOEs: one set is associated with the F20NH proton from the monomer; the other set is associated with the F20NH proton from the tetramer (Figure 2.8a). The monomer F20NH proton gives only an intraresidue
NOE to the F$_{20}$H$_{\alpha}$ proton. The tetramer F$_{20}$NH proton gives two NOEs associated with $\beta$-sheet folding: a stronger interresidue NOE to the F$_{10}$H$_{\alpha}$ proton and an intraresidue NOE to the F$_{20}$H$_{\alpha}$ proton. The tetramer F$_{20}$NH proton also gives an intermolecular NOE associated with interstrand interaction to the A$_{21}$H$_{\alpha}$ proton diagonally across the peptide dimer. This NOE is significant, because it reflects the dimer within the tetramer (Figure 2.9a). The tetramer F$_{20}$NH proton can not give an intermolecular NOE to the F$_{20}$NH proton diagonally across the peptide dimer, because the tetramer is symmetrical (Figure S2.7). Figure 2.9 summarizes the observed NOEs involving the $^{15}$NH protons between the dimers within the tetramer of peptide $[^{15}$N]1a.
Figure 2.8. $^{15}$N-Edited NOESY spectra of (a) peptide $^{[15N]}1a$ and (b) peptide $^{[15N]}1b$ at 8.0 mM in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K. The G$_{33}$H$_{α}$ corresponds to the pro-R $α$-proton and the G$_{33}$H$_{α}'$ corresponds to the pro-S $α$-proton. Crosspeaks associated with chemical exchange between the monomer and tetramer are labeled EX. $^{20}$
Figure 2.9. NOEs involving the $^{15}$NH protons between the dimers of peptides $[^{15}\text{N}]1\text{a}$ and $[^{15}\text{N}]1\text{b}$ within the respective tetramers. Blue and red arrows illustrate observed NOEs.

The $^{15}$N-edited NOESY spectrum of peptide $[^{15}\text{N}]1\text{b}$ also shows two sets of NOEs: one set is associated with the G$_{33}$NH proton from the monomer; the other set is associated with the G$_{33}$NH proton from the tetramer (Figure 2.8b). The monomer G$_{33}$NH proton gives a pattern of NOEs associated with β-sheet folding: two intraresidue NOEs to the diastereotopic G$_{33}$H$_{\alpha}$ and G$_{33}$H$_{\alpha'}$ protons and one interresidue NOE to the I$_{32}$H$_{\alpha}$ proton. The tetramer G$_{33}$NH proton also gives a pattern of NOEs associated with β-sheet folding: two intraresidue NOEs to the G$_{33}$H$_{\alpha}$ and G$_{33}$H$_{\alpha'}$ protons and one interresidue NOE to the I$_{32}$H$_{\alpha}$ proton. The tetramer G$_{33}$NH proton also gives an intermolecular NOE associated with interstrand interaction to the L$_{34}$H$_{\alpha}$ proton diagonally across the peptide dimer. This NOE is significant, because it reflects the dimer within the tetramer. The tetramer G$_{33}$NH proton can not give an intermolecular NOE to the G$_{33}$NH
proton diagonally across the peptide dimer, because the tetramer is symmetrical (Figure S2.8). Figure 2.9 summarizes the observed NOEs involving the $^{15}$NH protons between the dimers within the tetramer of peptide \([^{15}\text{N}]\text{1b}\).

**Molecular Models of the Tetramers.** I constructed energy-minimized models consistent with the observed NOEs to help understand the structures of the tetramers of peptides \(\text{1a}\) and \(\text{1b}\). I began with the X-ray crystallographic coordinates of a tetramer formed by a homologous macrocyclic $\beta$-sheet peptide (PDB ID: 3T4G).$^{10a}$ I mutated the side chains to the residues of peptides \(\text{1a}\) and \(\text{1b}\). I modified the alignment of the $\beta$-sheet dimers and oriented the dimers to reflect the observed NOEs. I then generated the minimum-energy models (local minima) of the tetramers. These models help illustrate the structures formed by the peptides derived from the central and C-terminal regions of A$\beta$. Figures 2.10 and 2.11 illustrate these models.

The energy-minimized model of the peptide \(\text{1a}\) tetramer consists of a $\beta$-sandwich of two four-stranded $\beta$-sheets that laminate together and form a hydrophobic core (Figure 2.10). The $\beta$-sheets exhibit a distinct twist that imparts a saddle shape. The side chains of L$_{17}$, F$_{19}$, and A$_{21}$ form a hydrophobic surface that packs in the hydrophobic core, while the side chains of E$_{22}$ and D$_{23}$ are exposed to solvent. The $\beta$-sheet dimers do not completely overlap, but rather are rotated roughly 30 degrees about the normal axis. The rotation and twist of the $\beta$-sheets allow the corners to pack tightly against each other. The corners of the $\beta$-sheet layers are nearly in contact, which is consistent with the observed interlayer NOEs between the Hao protons and the I$_{11}$ side-chain protons.
Figure 2.10. Molecular model of the tetramer formed by peptide 1a. (a) The tetramer with the side chains of L17, F19, A21, and D23 shown. (b) Dimer subunit of the tetramer with the side chains of L17, F19, A21, D23, I8, and I11 shown.

The energy-minimized model of the peptide 1b tetramer is similar to that of peptide 1a in that it also consists two four-stranded β-sheets that laminate together (Figure 2.11). The β-sheets are slightly less twisted, and the side chains of A30, I32, L34, and V36 form the hydrophobic surface that packs in the hydrophobic core. Like peptide 1a, the β-sheets are rotated roughly 30 degrees about the normal axis, allowing the corners to pack tightly against each other.
Figure 2.11. Molecular model of the tetramer formed by peptide 1b. (a) The tetramer with the side chains of A$_{30}$, I$_{32}$, L$_{34}$, and V$_{36}$ shown. (b) Dimer subunit of the tetramer with the side chains of A$_{30}$, I$_{32}$, L$_{34}$, V$_{36}$, I$_{8}$, and I$_{11}$ shown.
CONCLUSION

Macrocyclic β-sheet peptides 1 provide a platform with which to study the self-assembly of amyloid-derived peptides. Essential to the design of these β-sheet-forming peptides is the use of an amphiphilic template strand containing the peptide sequence IKHaoKI to block uncontrolled aggregation. The unnatural amino acid Hao promotes β-sheet formation and blocks uncontrolled hydrogen-bonding interactions. The Ile residues in the template strand give a distinct hydrophobic surface that promotes peptide assembly, while the Lys residues give a distinct hydrophilic surface that disfavors aggregation.

Incorporation of the central and C-terminal regions of Aβ into peptides 1 allows the study of these regions. The peptides containing these regions assemble through hydrogen-bonding and hydrophobic interactions to form β-sheet dimers that further assemble to form tetramers. NOESY and other 1H NMR studies show that the tetramers comprise a β-sandwich of two hydrogen-bonded dimers. Molecular modeling further elucidates the structures of the tetramers. The tetramers that form reflect the propensities of the central and C-terminal regions to assemble and adopt β-sheet structure.

Incorporation of a single 15N isotopic label into peptides 1 provides a spectroscopic probe that simplifies the spectra of the monomers and tetramers. 1H, 15N HSQC studies show that each peptide gives a single crosspeak associated with the monomer and a single crosspeak associated with the tetramer. 15N-Edited NOESY studies corroborate the pairing of the dimers within the tetramers. The hydrophobic amino acids Gly, Ala, Val, Leu, Ile, and Phe are widespread in amyloidogenic peptides and proteins and are readily available with an 15N isotopic label at reasonable cost. The incorporation of a single 15N-labeled amino acid as a spectroscopic probe promises to be broadly useful in studying the assembly and coassembly of peptides. In the accompanying chapter, I apply this approach to study the coassembly of peptides derived from the central and C-terminal regions of Aβ.
REFERENCES AND NOTES


16. $^1$H NMR studies were performed without buffer at pH 2.5 ± 0.5 to keep peptides $1\text{a}$ and $1\text{b}$ in the fully protonated state.


19. The ratio of diffusion coefficients of an oligomer and monomer reflects the oligomerization state as well as the shapes of the molecules. A tetramer will have a diffusion coefficient of about 0.59 to 0.63 times that of the monomer, while a dimer will have a diffusion coefficient of about 0.75 to 0.79 times that of the monomer. A tetramer could not easily be distinguished from a pentamer and could only marginally be distinguished from a trimer on the basis of diffusion coefficients measured by DOSY.
The observation of well-defined dimer subunits by NOESY in conjunction with the observed ratios of diffusion coefficients by DOSY clearly establishes the tetrameric state of the oligomers.

20. Chemical exchange between the monomer and tetramer of peptide \(1a\) is slow on the hundred-millisecond time scale at 298 K, but exchange increases at higher temperatures. An EXSY experiment shows a set of EXSY crosspeaks that indicate chemical exchange on the hundred-millisecond time scale at 318 K. (See the Supporting Information.) In contrast, chemical exchange between the monomer and tetramer of peptide \(1b\) occurs on the hundred-millisecond time scale even at 293 K.
SUPPORTING INFORMATION FOR

Assembly of Peptides Derived from β-Sheet Regions of

β-Amyloid
Figure S2.1. $^1$H NMR spectra of peptide 1a at various concentrations in D$_2$O at 600 MHz and 298 K. The 0.3 mM sample contains DSA as an internal standard, which is marked by an asterisk (*).
Figure S2.2. Expansions of the NOESY spectrum of peptide 1a at 8.0 mM in D$_2$O at 600 MHz and 298 K. Key NOEs associated with β-sheet folding and dimerization are highlighted in blue. The $^6$Orn pro-$R$ δ-protons are designated Ornδ$R$; The $^5$Orn pro-$R$ δ-protons are designated Ornδ$R$. 
Figure S2.3. Expansions of the NOESY spectrum of peptide 1a at 8.0 mM in D$_2$O at 600 MHz and 298 K. Key interlayer NOEs associated with tetramerization are highlighted in blue.
**Figure S2.4.** $^1$H NMR spectra of peptide 1b at various concentrations in D$_2$O at 600 MHz and 298 K. The 1.0 mM sample contains DSA as an internal standard, which is marked by an asterisk (*).
Figure S2.5. Expansions of the NOESY spectrum of peptide 1b at 16.0 mM in D$_2$O at 600 MHz and 293 K. Key NOEs associated with β-sheet folding and dimerization are highlighted in red. The G$_{33}$ pro-$S$ α-proton is designated G$_{33}$$\alpha$; the δOrn pro-$R$ δ-protons are designated Ornδ$_R$; The δOrn pro-$R$ δ-protons are designated Ornδ$_R$. 
Figure S2.6. Expansions of the NOESY spectrum of peptide 1b at 16.0 mM in D$_2$O at 600 MHz and 293 K. Key interlayer NOEs associated with tetramerization are highlighted in red.
Figure S2.7. $^{15}$N-Edited NOESY spectrum of peptide $[^{15}\text{N}]1\text{a}$ at 8.0 mM in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K.

Figure S2.8. $^{15}$N-Edited NOESY spectrum of peptide $[^{15}\text{N}]1\text{b}$ at 8.0 mM in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K. Crosspeaks associated with chemical exchange between the monomers and tetramers are labeled EX.
2.II. MATERIALS AND METHODS

General

\(N,N\)-Dimethylformamide (DMF), 2,4,6-collidine, and piperidine were purchased from Alfa Aesar and used without further purification. HPLC grade acetonitrile (CH\(_3\)CN) was purchased from VWR International and used without further purification. Methylene chloride (CH\(_2\)Cl\(_2\)) was purchased from Fisher Scientific, stored under argon, and passed through a column of alumina before use.\(^1\) Boc-Orn(Fmoc)-OH, HCTU, HBTU and HOBut were purchased from GL Biochem Ltd (Shanghai). 2-Chlorotrityl chloride resin and Fmoc protected amino acids were purchased from Chem-Impex International. \(N,N\)-Diisopropylethylamine (DIPEA), \(N\)-methylmorpholine (NMM), trifluoroacetic acid (TFA), and triisopropylsilane (TIPS) were purchased from Oakwood Chemical. Isotopically labeled glycine (\(^{15}\)N, 98%), phenylalanine (\(^{15}\)N, 98%), and deuterium oxide (D, 99.96%) were purchased from Cambridge Isotope Laboratories, Inc. Fmoc-Hao-OH was synthesized according to previously reported procedures.\(^2\)
Synthesis of Peptides 1

Resin Loading. 2-Chlorotrityl chloride resin (300 mg, 1.1 meq/g, 100–200 mesh) was suspended in ca. 8 mL of CH₂Cl₂ in a 10-mL Bio-Rad Poly-Prep column and allowed to swell (15 min). The CH₂Cl₂ was drained and a solution of Boc-Orn(Fmoc)-OH (0.22 mmol, 100.0 mg) in CH₂Cl₂ (7.6 mL) and 2,4,6-collidine (0.4 mL), was added. The suspension was agitated gently overnight (10–12 h) and the solution was drained. The capping solution 17:2:1 CH₂Cl₂/MeOH/DIPEA (8 mL) was added. The mixture was agitated gently (1 h), and then the solution was drained.
Solid-Phase Peptide Synthesis. The loaded resin was transferred to a solid-phase peptide synthesis vessel with DMF (3 × 2 mL). Successive rounds of solid-phase peptide synthesis were performed on a PS3™ Peptide Synthesizer (Protein Technologies) using the following conditions: The Fmoc deprotection steps (2 × 5 min) were performed with a 20% piperidine in DMF solution. The coupling steps (1 × 20 min) were performed for the amino acids (4 equiv) with HCTU (4 equiv) and a 20% 2,4,6-collidine in DMF solution. The unnatural amino acid Fmoc-Hao-OH (2 equiv) was coupled twice with 2 equiv of HCTU per coupling (60 min) to achieve complete coupling. DMF was used to rinse the resin after each deprotection (6 × 3 mL) and after each amino acid coupling (6 × 3 mL).

Cleavage from Resin. After the synthesis of each peptide was complete, the resin was transferred into the Poly-Prep column with CH2Cl2 (ca. 2 mL) and the solution was drained. The solid-phase peptide synthesis vessel was rinsed with ca. two additional portions of CH2Cl2 to ensure the complete transfer of the resin and the removal of DMF. A 1:4 HFIP/CH2Cl2 solution (8 mL) was added to the resin and the mixture was agitated gently. After 1 h, the solution was drained into a 250-mL round-bottom flask and the treatment with HFIP/CH2Cl2 solution was repeated. The combined solutions were evaporated under vacuum to give the protected linear peptides 1.

Cyclization. The protected linear peptides 1 were cyclized with HBTU (5 equiv), HOBt (5 equiv), and NMM (8 equiv) in a solution of DMF (125 mL). The solution was stirred under N2 overnight (12–24 h), and then the DMF was evaporated under vacuum. The peptides were placed under vacuum (ca. 0.1 mmHg) overnight to ensure complete removal of any residual DMF.
Deprotection. The protected cyclic peptides 1 were deprotected under acidic conditions with a solution of 18:1:1 TFA/triisopropylsilane/H₂O (10 mL). The solution was stirred for 2 h, then evaporated under vacuum. For peptides containing a methionine (1b and [¹⁵N]1b), 50 mg of dithiothreitol (DTT) was added to the solution to prevent sulfur oxidation.

RP-HPLC Purification. The peptides were suspended in a solution of 20% aqueous CH₃CN (ca. 8 mL) and the suspensions were filtered through a 0.2 µm filter. The purity of each peptide was analyzed by analytical RP-HPLC on a Phenomenex Aeries 2.6µ XB-C18 column (150 mm x 4.6 mm) with a 5–100% gradient over 20 min of CH₃CN in H₂O with 0.1% TFA at 1.0 mL/min. The purification of each peptides was performed by preparative RP-HPLC on an Agilent Zorbax 7 µM SB-C18 Prep HT column (21.2 mm x 250 mm) with a 15–30% gradient over 10 min and 30–60% gradient over 45 min of CH₃CN in H₂O with 0.1% TFA at 15.0 mL/min. The pure fractions were combined and concentrated under vacuum. The peptides were re-suspended in a solution of H₂O with 0.1% TFA (ca. 10–15 mL), then lyophilized to give peptides 1 as a white powder in 8–22% yield (30–80 mg) based on the resin loading of the first amino acid Boc-Orn(Fmoc)-OH).
Fmoc-Protection of $^{15}$N-Labeled Amino Acids

Fmoc-$[^{15}$N]$\text{Phe-OH}$: A 100-mL one-neck round-bottom flask equipped with a magnetic stirring bar was charged with $^{15}$N-labeled phenylalanine (1.0 g, 6 mmol) and a solution of 1:1 CH$_3$CN/H$_2$O (50 mL). Et$_3$N (0.6 g, 6 mmol) and Fmoc-OSu (1.9 g, 5.7 mmol) were added, then the reaction mixture was stirred until the solution turned clear (ca. 15 min). Additional Et$_3$N was added until the pH was roughly 8.5, then the mixture was stirred for 1 h. The mixture was poured into a solution of 1.0 M HCl (250 mL) in a 400-mL beaker while stirring vigorously. The Fmoc-$[^{15}$N]$\text{Phe-OH}$ precipitated from the solution and the solid was isolated by filtering the mixture through a sintered glass filter funnel with a medium frit. The funnel was covered with a piece of filter paper and the solid was dried by aspirating air through the funnel. The solid was suspended in ca. 200 mL of EtOAc to form a turbid solution. The solution was stirred vigorously for 10 min, dried over MgSO$_4$, filtered, and then concentrated under vacuum to give a white solid. The isolated solid was ground into a fine powder to give ca. 1.94 g (92%). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.77 (d, $J = 7.5$ Hz, 2H), 7.55 (t, $J = 6.2$ Hz, 2H), 7.40 (t, $J = 7.4$ Hz, 2H), 6.80 (m, 5H), 7.15 (d, $J = 6.6$ Hz, 2H), 5.19 (dd, $J = 91.9$, 8.2 Hz, 1H), 4.70 (m, 1H), 4.46 (dd, $J = 10.4$, 7.3 Hz, 1H), 4.37 (t, $J = 8.7$ Hz, 1H), 4.21 (t, $J = 6.7$ Hz, 1H), 3.18 (m, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 175.2, 156.0 (d, $^1J_{\text{CN}} = 25$ Hz), 144.0, 141.6, 135.6, 129.6, 129.0, 128.0, 127.6, 127.3, 125.3, 120.3, 67.3, 54.7 (d, $^1J_{\text{CN}} = 13.8$ Hz), 47.4, 37.9, 30.0.
Fmoc-\[^{15}\text{N}\]Gly-OH: A 100-mL one-neck round-bottom flask equipped with a magnetic stirring bar was charged with \(^{15}\text{N}\)-labeled glycine (1.0 g, 13 mmol) and a solution of 1:1 CH\(_3\)CN/H\(_2\)O (50 mL). Et\(_3\)N (1.3 g, 13 mmol) and Fmoc-OSu (4.2 g, 12.5 mmol) were added, then the reaction mixture was stirred until the solution turned clear (ca. 15 min). Additional Et\(_3\)N was added until the pH was roughly 8.5, then the mixture was stirred for 1 h. The mixture was poured into a solution of 1.0 M HCl (250 mL) in a 400-mL beaker while stirring vigorously. The Fmoc-\[^{15}\text{N}\]Gly-OH precipitated from the solution and the solid was isolated by filtering the mixture through a sintered glass filter funnel with a medium frit. The funnel was covered with a piece of filter paper and the solid was dried by aspirating air through the funnel. The solid was suspended in ca. 200 mL of EtOAc to form a turbid solution. The solution was stirred vigorously for 10 min, dried over MgSO\(_4\), filtered, and then concentrated under vacuum to give a white solid. The isolated solid was ground into a fine powder to give ca. 3.48 g (92%) isolated yield. \(^1\text{H}\) NMR (500 MHz, CDCl\(_3\)): \(\delta\) 7.77 (d, \(J = 7.5\) Hz, 2H), 7.60 (d, \(J = 7.4\) Hz, 2H), 7.40 (t, \(J = 7.5\) Hz, 2H), 7.32 (t, \(J = 7.5\), 1 Hz, 2H), 5.28 (dt \(J = 9.2.6, 5.6\) Hz), 4.43 (d, \(J = 7.0\) Hz, 2H), 4.24 (t, \(J = 7.0\) Hz, 1H), 4.04 (d, 5.5 Hz, 2H); \(^{13}\text{C}\) NMR (125 MHz, CDCl\(_3\)): \(\delta\) 173.6, 156.5, 144.0, 141.6, 128.0, 127.3, 125.3, 120.3, 67.6, 47.3, 42.6 (d, \(J_{\text{CN}} = 13.8\) Hz).
NMR Spectroscopy of Peptides 1

Sample Preparation. NMR spectroscopy of peptides 1a and 1b was performed in D$_2$O. The solutions were prepared by dissolving a weighed portion of the peptide in the appropriate volume of solvent. The molecular weights of the peptides were calculated as the TFA salts with all amino groups assumed to be protonated (1a, M.W. 2223.85 g/mol and 1b, M.W. 2099.91 g/mol). The solutions were allowed to stand for 24 h to allow complete hydrogen to deuterium exchange of the amide NH protons.

$^1$H NMR, TOCSY, ROESY, and NOESY Data Collection. NMR spectra were recorded on a Bruker 600 MHz spectrometer with a TBI probe. Presaturation water suppression was applied as needed. TOCSY spectra were recorded with 2048 points in the $f_2$ dimension and 512 increments in the $f_1$ dimension with a 150-ms spin-lock mixing time. ROESY spectra were recorded with 2048 points in the $f_2$ dimension and 512 increments in the $f_1$ dimension with a 200-ms spin-lock mixing time. NOESY spectra were recorded with 2048 points in the $f_2$ dimension and 512 increments in the $f_1$ dimension with a 150-ms mixing time.

$^1$H NMR, TOCSY, ROESY, and NOESY Data Processing. NMR spectra were processed with Bruker XwinNMR software. Automatic baseline correction was applied in both dimensions after phasing the spectra. TOCSY and ROESY spectra were Fourier transformed to a final matrix size of 2048 x 1024 real points using a Qsine weighting function and forward linear prediction. NOESY spectra were Fourier transformed to a final matrix size of 2048 x 2048 real points using a Qsine weighting function and forward linear prediction.
**Diffusion-Ordered Spectroscopy (DOSY) Experiments.** DOSY experiments were performed on a Bruker 500 MHz spectrometer equipped with a TCI cryoprobe, with a diffusion delay (Δ) of 75-ms and a diffusion gradient length (δ) of 2.5-ms. Sixteen sets of FIDs were recorded with the gradient strength incremented from 5%–95% using a linear ramp. The combined FIDs were Fourier transformed in Bruker's TopSpin™ software to give a pseudo-2D spectrum. After phasing and performing baseline correction, each pseudo-2D spectrum was processed with logarithmic scaling on the Y-axis. The Y-axis was calibrated to the diffusion coefficient of the residual HOD peak in D2O (1.9 x 10⁻⁹ m²/s at 298 K). The diffusion coefficients of the peptides were read and converted from logarithmic values to linear values.

**NMR Spectroscopy of Peptides [¹⁵N]1**

*Sample Preparation.* NMR spectroscopy of peptides [¹⁵N]1a and [¹⁵N]1b was performed in 9:1 H₂O/D₂O. The solutions were prepared by dissolving a weighed portion of the peptide in the appropriate volume of solvent. The molecular weights of the peptides were calculated as the TFA salts with all amino groups assumed to be protonated ([¹⁵N]1a, M.W. 2224.85 g/mol and [¹⁵N]1b, M.W. 2100.91 g/mol). 4,4-Dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA) was added as an internal standard for referencing chemical shifts.
$^1$H NMR, $^1$H,$^{15}$N HSQC, and $^1$H,$^{15}$N NOESY-HSQC ($^{15}$N-edited NOESY) Data Collection. NMR spectra were recorded on a Bruker 600 MHz spectrometer with either a TBI probe or a BBFO cryoprobe. Gradient water suppression was applied as needed. $^1$H,$^{15}$N HSQC spectra were recorded with 1024 points in the $f_2$ dimension and 512 increments in the $f_1$ dimension. $^1$H,$^{15}$N NOESY-HSQC spectra were recorded with a 150-ms mixing time, and with 2048 points in the $f_3$ dimension ($^1$H), 1 increment in the $f_2$ dimension ($^{15}$N), and 1024 increments in the $f_1$ dimension ($^1$H).

$^1$H NMR, $^1$H,$^{15}$N HSQC, and $^1$H,$^{15}$N NOESY-HSQC ($^{15}$N-edited NOESY) Data Processing. NMR spectra were Fourier transformed in Bruker XwinNMR software with forward linear prediction and a Qsinc weighting function. Automatic baseline correction was applied in both dimensions after phasing the spectra. The $^1$H,$^{15}$N HSQC spectra were processed to a final matrix size of 2048 x 1024 real points and with GB = 0.1 in the $f_2$ dimension. The $^1$H,$^{15}$N NOESY-HSQC spectra were processed to a final 2D matrix size of 4096 x 2048 real points ($f_3,f_1$) and with GB = 0.05 in both dimensions.
**Molecular Modeling of Peptides 1a and 1b.**

Molecular models of the tetramers of peptides 1a and 1b were generated from the X-ray crystallographic structure of a similar macrocyclic β-sheet peptide (PDB 3T4G). This peptide contains AIIGLMV (Aβ30–36) in the heptapeptide strand and KFFBrK in positions R8-R11 in the template strand. The PDB coordinates were imported into PyMOL. Symmetry mates were generated to create two hydrogen-bonded dimers sandwiched on the surface displaying the side chains of A30, I32, L34, and V36. The alignment of each dimer was shifted by two residues to match the alignment of the dimers of peptides 1a and 1b. The residues of the dimers were mutated to match peptide 1a or peptide 1b, and the side chain torsion angles of χ1 and χ2 were adjusted for Ile (180° and 60°) and Phe (180°). The dimers were then rotated manually to reflect the observed interlayer NOEs between Ile11 and the methoxy group of Hao.

The coordinates were exported from PyMOL as a .pdb file. The file was imported into MacroModel with the Maestro user interface. Atom types and bond orders were edited as needed to correct errors in bond type and charge. Distance constraints were applied to reflect the folding and dimerization of the macrocycles. Four interlayer distance constraints between the δ-methyl group of Ile11 and the methoxy group of Hao were applied to reflect the observed interlayer contacts. Minimization was performed with the MMFFs force field and GB/SA water solvation. All constraints were removed and minimization was repeated to generate a minimum-energy conformation (local minimum). The coordinates were exported in .pdb file format and imported into PyMOL.
2.III. REFERENCES


2.IV. CHARACTERIZATION DATA
RP-HPLC of peptide 1a

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</table>

Totals:

2.5083e4 1820.18323

MS (ESI) of peptide 1a

NT_iv_1a-1 24 (0.440) Sb (1,10.00); 5m (Mn, 4x3.00); Cm (23:31)

calculated m/z for C_{85}H_{130}N_{20}O_{21}:

[M+H]^+: 1767.98
[2M+3H]^3+: 1178.98
[M+2H]^2+: 884.49
[M+3H]^3+: 590.00

Column: Aeris XB-C18 2.6 μ
Dimensions: 150 mm x 4.6 mm
Mobile phase: A: H2O, 0.1% TFA
B: CH3CN, 0.1% TFA
Gradient: A/B (95:5) to (0:100) in 20 min
Flow rate: 1.0 mL/min
Detection: VWD, wavelength = 214 nm
Temperature: 298 K

TOF MS ES+
1.44e3
$^1\text{H NMR of peptide 1a, 0.15 mM in D}_2\text{O at 600 MHz and 293 K}$
$^1$H NMR of peptide 1a, 0.15 mM in D$_2$O at 600 MHz and 293 K
$^1$H NMR of peptide 1a, 0.15 mM in D$_2$O at 600 MHz and 293 K
$^1$H NMR of peptide 1a, 0.15 mM in D$_2$O at 600 MHz and 293 K
$^1$H NMR of peptide 1a, 0.15 mM in D$_2$O at 600 MHz and 293 K
\( ^1H \) NMR of peptide 1a, 0.15 mM in D\(_2\)O at 600 MHz and 293 K

\[
\begin{align*}
| & ppm | 1.8663 & 1.8194 & 1.7624 & 1.7194 & 1.7068 & 1.6946 & 1.6142 & 1.5283 & 1.3756 & 1.3354 & 1.3240 & 1.2231 & 1.1522 & 1.0431 & 1.0344 & 0.9884 & 0.9379 & 0.9195 & 0.9102 & 0.8984 & 0.8856 & 0.8730 & 0.8634 & 0.8452 & 0.8014 & 0.7291 & 0.7201 & 0.6265 | \\
& \text{Integral} | 56.826 & 15.306 & 61.324 & \text{ppm} | 2.0582 & 8 & 2.0205 & 2 & 1.8663 & 3 & 1.7624 & 2 & 1.7194 & 2 & 1.7068 & 9 & 1.6946 & 1 & 1.6142 & 2 & 1.5283 & 3 & 1.3756 & 8 & 1.3354 & 4 & 1.3240 & 4 & 1.2231 & 5 & 1.1522 & 5 & 1.0431 & 8 & 1.0344 & 7 & 0.9884 & 8 & 0.9379 & 9 & 0.9195 & 9 & 0.9102 & 7 & 0.8984 & 3 & 0.8856 & 3 & 0.8730 & 6 & 0.8634 & 3 & 0.8452 & 4 & 0.8014 & 5 & 0.7291 & 9 & 0.7201 & 5 & 0.6265 & 0 | \\
\end{align*}
\]

Current Data Parameters

**USER             ntruex**

**NAME     NT_iv_130_1a_0.15mM_p**

**EXPNO** 11

**PROCNO** 1

**F2 - Acquisition Parameters**

**Date_** 20160423

**Time** 21.26

**INSTRUM** av600

**PROBHD** 5 mm CPBBO BB-

**PULPROG** zg30

**TD** 97922

**SOLVENT** D\(_2\)O

**NS** 512

**DS** 2

**SWH** 9615.385 Hz

**FIDRES** 0.098194 Hz

**AQ** 5.0919938 sec

**RG** 12.7

**DW** 52.000 usec

**DE** 13.70 usec

**TE** 293.0 K

**D1** 0.10000000 sec

**TD0** 1

**======== CHANNEL f1 ========**

**SFO1** 600.1342009 MHz

**NUC1** 1H

**P1** 12.00 usec

**F2 - Processing parameters**

**SI** 65536

**SF** 600.1299881 MHz

**WDW** EM

**SSB** 0

**LB** 1.00 Hz

**GB** 0

**PC** 1.00

1D NMR plot parameters

**CX** 22.80 cm

**CY** 15.00 cm

**F1P** 2.100 ppm

**F1** 1260.27 Hz

**F2P** 0.300 ppm

**F2** 180.04 Hz

**PPMCM** 0.07895 ppm/cm

**HZCM** 47.37869 Hz/cm
$^1$H NMR 2D TOCSY of peptide 1a with presaturation suppression of the HOD peak
0.15 mM in D$_2$O at 600 MHz and 293 K with 150-ms spin-lock mixing time
$^1$H NMR 2D TOCSY of peptide 1a with presaturation suppression of the HOD peak
0.15 mM in D$_2$O at 600 MHz and 293 K with 150-ms spin-lock mixing time
$^1$H NMR 2D ROESY of peptide 1a with presaturation suppression of the HOD peak 0.15 mM in D$_2$O at 600 MHz and 293 K with 200-ms spin-lock mixing time.
$^1$H NMR 2D ROESY of peptide 1a with presaturation suppression of the HOD peak 0.15 mM in D$_2$O at 600 MHz and 293 K with 200-ms spin-lock mixing time
$^1$H NMR DOSY of peptide 1a, 0.15 mM in D$_2$O at 500 MHz and 298 K

Calculations for peptide 1a at 0.15 mM

$D_{HOD} = 19.0 \times 10^{-10}$ m$^2$/s $^a$

$log(D_{HOD}) = -8.721$

$D_{monomer}$: log($D$) = -9.69; $D = 10^{-9.69} = 20.4 \pm 1.7 \times 10^{-11}$ m$^2$/s

$D_{tetramer}$: log($D$) = -9.90; $D = 10^{-9.90} = 12.6 \pm 1.6 \times 10^{-11}$ m$^2$/s

$^1$H NMR of peptide 1a, 8 mM in D$_2$O at 600 MHz and 298 K

Current Data Parameters

**USER**

**NAME**     NT_iii_112_1a_8mM_D2O
**EXPNO**                21
**PROCNO**                1

**F2 - Acquisition Parameters**

**Date_**          20150825
**Time**              21.12
**INSTRUM**           av600
**PROBHD**   5 mm TBI 1H/13
**PULPROG**            zg30
**TD**                97922
**SOLVENT**             D2O
**NS**                    8
**DS**                    2
**SWH**            9615.385 Hz            **FIDRES**         0.098194 Hz
**AQ**            5.0919938 sec
**RG**                  181
**DW**               52.000 usec
**DE**                14.54 usec
**TE**                298.0 K
**D1**           0.10000000 sec
**TD0**                   1

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**SFO1**        600.1342009 MHz
**NUC1**                 1H
**P1**                 8.00 usec

**F2 - Processing parameters**

**SI**                65536
**SF**          600.1300240 MHz
**WDW**                  EM
**SSB**                   0
**LB**                 0.30 Hz
**GB**                    0
**PC**                 1.00

**1D NMR plot parameters**

**CX**                22.80 cm
**CY**                15.00 cm
**F1P**               8.500 ppm
**F1**              5101.10 Hz
**F2P**               0.300 ppm
**F2**               180.04 Hz

**PPMCM**           0.35965 ppm/cm
**HZCM**          215.83623 Hz/cm

...
$^1$H NMR of peptide 1a, 8 mM in D$_2$O at 600 MHz and 298 K

[Diagram of the peptide structure]
$^1$H NMR of peptide 1a, 8 mM in D$_2$O at 600 MHz and 298 K

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**Current Data Parameters**

**USER**

**NAME**

**EXPNO**

**PROCNO**

**F2 - Acquisition Parameters**

- **Date**: 20150825
- **Time**: 21.12
- **INSTRUM**: av600
- **PROBHD**: 5 mm TBI 1H/13
- **PULPROG**: zg30
- **TD**: 97922
- **SOLVENT**: D$_2$O
- **NS**: 8
- **DS**: 2
- **SWH**: 9615.385 Hz, **FIDRES**: 0.098194 Hz
- **AQ**: 5.0919938 sec
- **RG**: 181
- **DW**: 52.000 usec
- **DE**: 14.54 usec
- **TE**: 298.0 K
- **D1**: 0.10000000 sec
- **TD0**: 1

**F1 - Processing parameters**

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- **SF**: 600.1300240 MHz
- **WDW**: EM
- **SSB**: 0
- **LB**: 0.30 Hz
- **GB**: 0
- **PC**: 1.00

**1D NMR plot parameters**

- **CX**: 22.80 cm
- **CY**: 15.00 cm
- **F1P**: 7.500 ppm
- **F1**: 4500.98 Hz
- **F2P**: 5.800 ppm
- **F2**: 3480.75 Hz
- **PPMCM**: 0.07456 ppm/cm
- **HZCM**: 44.74653 Hz/cm
$^1$H NMR of peptide 1a, 8 mM in D$_2$O at 600 MHz and 298 K
$^1$H NMR of peptide 1a, 8 mM in D$_2$O at 600 MHz and 298 K

[Image of NMR spectrum and structure of peptide 1a]

Current Data Parameters

USER             ntruex
NAME     NT_iii_112_1a_8mM_D2O
EXPNO                21
PROCNO                1

F2 - Acquisition Parameters
Date_          20150825
Time              21.12
INSTRUM           av600
PROBHD   5 mm TBI 1H/13
PULPROG            zg30
TD                97922
SOLVENT             D2O
NS                    8
DS                    2
SWH            9615.385 Hz            FIDRES         0.098194 Hz
AQ            5.0919938 sec
RG                  181
DW               52.000 usec
DE                14.54 usec
TE                298.0 K
D1           0.10000000 sec
TD0                   1

======== CHANNEL f1 ========
SFO1        600.1342009 MHz
NUC1                 1H
P1                 8.00 usec

F2 - Processing parameters
SI                65536
SF          600.1300240 MHz
WDW                  EM
SSB                   0
LB                 0.30 Hz
GB                    0
PC                 1.00

1D NMR plot parameters
CX                22.80 cm
CY                15.00 cm
F1P               4.000 ppm
F1              2400.52 Hz
F2P               2.300 ppm
F2              1380.30 Hz
PPMCM           0.07456 ppm/cm
HZCM           44.74654 Hz/cm
1H NMR of peptide 1a, 8 mM in D2O at 600 MHz and 298 K
$^1$H NMR 2D TOCSY of peptide 1a with presaturation suppression of the HOD peak
8 mM in D$_2$O at 600 MHz and 298 K with 150-ms spin-lock mixing time.
$^{1}$H NMR 2D TOCSY of peptide 1a with presaturation suppression of the HOD peak
8 mM in D$_2$O at 600 MHz and 298 K with 150-ms spin-lock mixing time
$^1$H NMR 2D TOCSY of peptide 1a with presaturation suppression of the HOD peak
8 mM in D$_2$O at 600 MHz and 298 K with 150-ms spin-lock mixing time
$^1$H NMR 2D NOESY of peptide 1a with presaturation suppression of the HOD peak
8 mM in D$_2$O at 600 MHz and 298 K with 150-ms mixing time
$^1$H NMR 2D NOESY of peptide 1a with presaturation suppression of the HOD peak
8 mM in D$_2$O at 600 MHz and 298 K with 150-ms mixing time
$^1$H NMR 2D NOESY of peptide $1a$ with presaturation suppression of the HOD peak
8 mM in D$_2$O at 600 MHz and 298 K with 150-ms mixing time
$^1$H NMR 2D NOESY of peptide 1a with presaturation suppression of the HOD peak
8 mM in D$_2$O at 600 MHz and 298 K with 150-ms mixing time
$^1$H NMR 2D EXSY of peptide 1a with presaturation suppression of the HOD peak
8 mM in D$_2$O at 600 MHz and 318 K with 200-ms spin-lock mixing time
Exchange crosspeaks are shown in red;
ROE crosspeaks are shown in black.
$^1$H NMR 2D EXSY of peptide 1a with presaturation suppression of the HOD peak
8 mM in D$_2$O at 600 MHz and 318 K with 200-ms spin-lock mixing time
Exchange crosspeaks are shown in red;
ROE crosspeaks are shown in black.
$^1$H NMR 2D EXSY of peptide 1a with presaturation suppression of the HOD peak 8 mM in D$_2$O at 600 MHz and 318 K with 200-ms spin-lock mixing time. Exchange crosspeaks are shown in red; ROE crosspeaks are shown in black.
\( ^1 \text{H NMR DOSY of peptide 1a, 8 mM in D}_2\text{O at 500 MHz and 298 K} \)

tetramer predominates

Calculations for peptide 1a at 8.0 mM

\( D_{\text{HOD}} = 19.0 \times 10^{-10} \text{ m}^2/\text{s} \)
\[ \log(D_{\text{HOD}}) = -8.721 \]

\( D_{\text{tetramer}} \cdot \log(D) = -9.928 \cdot D = 10^{-9.928} = 11.8 \pm 1.0 \times 10^{-11} \text{ m}^2/\text{s} \)

RP-HPLC of peptide 1b

MS (ESI) of peptide 1b

NT_iv_1b-1 6 (0.110) Sb (1,10.00 ); Sm (Mn, 4x3.00); Cm (6:26)

-[M+H]+: 1644.00
-[2M+3H]3+: 1096.33
-[M+2H]2+: 822.51
-[M+3H]3+: 548.67
NT Iv 1b-1 6 (0.110) Sb (1,10.00); Sm (Mn, 4x3.00); Cm (6:26) TOF MS ES+

\[ [M+H]^+ \]
\[ [2M+4H]^2+ \]

\[ [2M+3H]^3+ \]
\[ [4M+6H]^6+ \]
$^1$H NMR of peptide 1b, 1 mM in D$_2$O at 600 MHz and 293 K
$^1$H NMR of peptide 1b, 1 mM in D$_2$O at 600 MHz and 293 K

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**PROCNO** 1

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FIDRES 0.098194 Hz
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RG 322
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DE 14.54 usec
TE 292.9 K
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**CHANNEL f1**
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NUC1 1H
P1 8.00 usec

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---

**Chemical Structures**

- HN
- HO
- HN
- NO
- ...
$^1$H NMR of peptide 1b, 1 mM in D$_2$O at 600 MHz and 293 K

Current data parameters

- Date: 20160121
- Time: 20.50
- Instrument: av600
- Probe: 5 mm TBI 1H/13C

F2 - Acquisition parameters

- TD: 97922
- solvent: D$_2$O
- NS: 32
- DS: 2
- SWH: 9615.385 Hz
- FIDRES: 0.098194 Hz
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1H NMR of peptide 1b, 1 mM in D2O at 600 MHz and 293 K
$^1$H NMR of peptide 1b, 1 mM in D$_2$O at 600 MHz and 293 K
$^1$H NMR 2D TOCSY of macrocycle 1b with presaturation suppression of the HOD peak
1 mM in D$_2$O at 600 MHz and 293 K with 150-ms spin-lock mixing time
$^1$H NMR 2D TOCSY of macrocycle 1b with presaturation suppression of the HOD peak
1 mM in D$_2$O at 600 MHz and 293 K with 150-ms spin-lock mixing time
$^1$H NMR 2D ROESY of macrocycle 1b with presaturation suppression of the HOD peak
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$^1$H NMR 2D ROESY of macrocycle 1b with presaturation suppression of the HOD peak
1 mM in D$_2$O at 600 MHz and 293 K with 200-ms spin-lock mixing time
$^1$H NMR DOSY of peptide 1b, 1 mM in D$_2$O at 500 MHz and 298 K
monomer predominates

Calculations for peptide 1b at 1.0 mM

$D_{HOD} = 19.0 \times 10^{-10}$ m$^2$/s $^a$

$log (D_{HOD}) = -8.721$

$D_{monomer}: log(D) = -9.712; D = 10^{-9.712} = 19.4 \pm 1.7 \times 10^{-11}$ m$^2$/s

$^1$H NMR of peptide 1b, 16 mM in D$_2$O at 600 MHz and 293 K

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1H NMR of peptide 1b, 16 mM in D$_2$O at 600 MHz and 293 K.
$^1$H NMR of peptide 1b, 16 mM in D$_2$O at 600 MHz and 293 K
$\text{H NMR of peptide 1b, 16 mM in D}_2\text{O at 600 MHz and 293 K}$

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PROCNO                1
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1D NMR plot parameters
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\[
\text{H NMR of peptide 1b, 16 mM in D}_2\text{O at 600 MHz and 293 K}
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$^1$H NMR of peptide 1b, 16 mM in D$_2$O at 600 MHz and 293 K
$^1$H NMR of peptide 1b, 16 mM in D$_2$O at 600 MHz and 293 K
$^1$H NMR 2D TOCSY of macrocycle 1b with presaturation suppression of the HOD peak 16 mM in D$_2$O at 600 MHz and 293 K with 150-ms spin-lock mixing time
$^1$H NMR 2D TOCSY of macrocycle 1b with presaturation suppression of the HOD peak
16 mM in D$_2$O at 600 MHz and 293 K with 150-ms spin-lock mixing time
$^1$H NMR 2D TOCSY of macrocycle 1b with presaturation suppression of the HOD peak 16 mM in D$_2$O at 600 MHz and 293 K with 150-ms spin-lock mixing time
$^1$H NMR 2D NOESY of macrocycle 1b with presaturation suppression of the HOD peak 16 mM in D$_2$O at 600 MHz and 293 K with 150-ms mixing time
$^1$H NMR 2D NOESY of macrocycle 1b with presaturation suppression of the HOD peak
16 mM in D$_2$O at 600 MHz and 293 K with 150-ms mixing time
$^1$H NMR 2D NOESY of macrocycle 1b with presaturation suppression of the HOD peak
16 mM in D$_2$O at 600 MHz and 293 K with 150-ms mixing time
$^1$H NMR DOSY of peptide 1b, 16 mM in D$_2$O at 500 MHz and 298 K tetramer predominates

Calculations for peptide 1b at 16.0 mM

$D_{\text{HOD}} = 19.0 \times 10^{-10}$ m$^2$/s \( a \)

$log (D_{\text{HOD}}) = -8.721$

$D_{\text{tetramer}} \cdot log(D) = -9.924; D = 10^{-9.924} = 11.9 \pm 1.1 \times 10^{-11}$ m$^2$/s

$^1$H NMR of Fmoc-$^{15}$N-Phe-OH in CDCl$_3$ at 500 MHz and 298 K
\textbf{1H NMR of Fmoc-[^{15}N]Phe-OH in CDCl\textsubscript{3} at 500 MHz and 298 K}
$^1$H NMR of Fmoc-$[^{15}$N]Gly-OH in CDCl$_3$ at 500 MHz and 298 K
### 13C NMR of Fmoc-[15N]Gly-OH in CDCl₃ at 500 MHz and 298 K

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- **WDW**: EM
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- **PC**: 1.00

**1D NMR plot parameters**

- **CX**: 22.80 cm
- **CY**: 25.00 cm
- **F1P**: 200.000 ppm
- **F1**: 25156.08 Hz
- **F2P**: 20.000 ppm
- **F2**: 2515.61 Hz
- **PPMCM**: 7.89474 ppm/cm
- **HZCM**: 993.00317 Hz/cm
$^{1}H,^{15}N$ HSQC of peptide $[^{15}N]1a$ in 9:1 $H_2O/D_2O$ at 600 MHz and 293 K
8.0 mM total concentration
$^1$H,$^{15}$N HSQC of peptide $[^{15}$N]$^1$b in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K
8.0 mM total concentration
CHAPTER 3

Coassembly of Peptides Derived from β-Sheet Regions of β-Amyloid
INTRODUCTION

Interactions among β-sheets are critical in the aggregation of the β-amyloid peptide (Aβ) to form oligomers and fibrils in Alzheimer’s disease.\(^1\) Two regions of the 40- or 42-residue peptide adopt β-sheet structure and promote aggregation: the central region and the C-terminal region.\(^2\) The central region comprises the hydrophobic pentapeptide LVFFA (Aβ\(_{17-23}\)), and the C-terminal region comprises the hydrophobic undecapeptide AIIGLMVGGVV (Aβ\(_{30-40}\)) or the hydrophobic tridecapeptide AIIGLMVGGVVIA (Aβ\(_{30-42}\)).

Elucidating the roles of the central and C-terminal regions of Aβ is critical to understanding Aβ aggregation. These two regions assemble differently in the fibrils and in the toxic oligomers that cause synaptic dysfunction and cell death. In Aβ\(_{1-40}\) fibrils, the peptide forms parallel β-sheets, with the central and C-terminal regions laminated together.\(^3,4\) In the oligomers, the peptide is thought to form β-hairpins comprising antiparallel β-sheets.\(^5\)

In the preceding chapter, I incorporated residues from the central and C-terminal regions into macrocyclic β-sheet peptides 1, and I determined how the peptides assembled in aqueous solution.\(^6\) Peptides 1 consist of a heptapeptide strand, a template strand containing the unnatural amino acid Hao, and two δ-linked ornithine turn units.\(^7,8,9\) I incorporated residues LVFFAED (Aβ\(_{17-23}\)) and residues AIIGLMV (Aβ\(_{30-36}\)) into the heptapeptide strands of peptides 1\(_a\) and 1\(_b\), respectively. I incorporated isoleucine residues (I\(_8\) and I\(_{11}\)) into the template strand to promote assembly and lysine residues (K\(_9\) and K\(_{10}\)) to maintain solubility.\(^1\)H NMR studies of peptides 1\(_a\) and 1\(_b\) show that the peptides assemble to form sandwich-like homotetramers, consisting of two hydrogen-bonded dimers.
Incorporation of a single isotopic label into peptides 1 facilitated the identification and quantification of the tetramers. The peptides [15N]1a and [15N]1b each contain a single 15N-labeled amino acid in the center of the heptapeptide strand. Peptide [15N]1a contains an 15N label in the F20 residue; peptide [15N]1b contains an 15N label in the G33 residue. 1H,15N HSQC studies show only the resonances associated with the 15N label, reducing each spectrum to two crosspeaks: The 1H,15N HSQC spectrum of peptide [15N]1a shows one crosspeak associated with the monomer and another associated with the homotetramer. The 1H,15N HSQC spectrum of peptide [15N]1b also shows one crosspeak associated with the monomer and another associated with the homotetramer.
In this chapter, I ask whether these peptides prefer to coassemble or to segregate. To address this question, I mix peptides 1a and 1b and characterize the oligomers that form. H NMR studies show that peptides 1a and 1b form a mixture of homotetramers and heterotetramers, but the H NMR spectrum of the mixture is largely indecipherable. To characterize the complex mixture of homotetramers and heterotetramers, I use the 15N-labeled peptides [15N]1a and [15N]1b and H, 15N NMR spectroscopy. H, 15N HSQC, in conjunction with Job’s method of continuous variation, reveals that the peptides form three heterotetramers in 3:1, 2:2, and 1:3 stoichiometries, in addition to the two homotetramers. The following describes the characterization of these five tetramers and the equilibria among them.

RESULTS AND DISCUSSION

Peptides 1a and 1b Coassemble upon Mixing. The H NMR spectrum of pure peptide 1a at 8.0 mM predominately shows the homotetramer; the H NMR spectrum of pure peptide 1b at 8.0 mM shows the monomer and the homotetramer. In a 1:1 mixture of peptides 1a and 1b at 8.0 mM total concentration, the H NMR spectrum shows many new resonances: The resonances from the homotetramer of peptide 1a diminish greatly and the resonances from the homotetramer of peptide 1b nearly disappear. New resonances appear in the spectrum in the aromatic region.
between 6 and 9 ppm and also in the methyl region below 1 ppm. Several new Hao methoxy (HaoOMe) resonances appear between 4 and 4.5 ppm. The HaoOMe resonance from the homotetramer of peptide 1a diminishes greatly and the HaoOMe resonance from the homotetramer of peptide 1b almost completely disappears. The multitude of new resonances in the spectrum of the 1:1 mixture suggests that several new oligomers form, rather than just one. Figure 3.1 shows the $^1$H NMR spectra of pure 1a, pure 1b, and the 1:1 mixture.

**Figure 3.1.** $^1$H NMR spectra of (a) peptide 1a at 8.0 mM, (b) peptide 1b at 8.0 mM, and (c) the 1:1 mixture of peptides 1a and 1b at 8.0 mM total concentration in D$_2$O at 600 MHz and 298 K. Dotted lines illustrate how the resonances from the 1:1 mixture compare with the resonances of pure 1a and pure 1b.
**Peptides 1a and 1b Form Heterotetramers.** Our laboratory has previously shown that related macrocyclic β-sheets can assemble to form tetramers. In the preceding chapter, I established that both peptide 1a and peptide 1b form tetramers by measuring the diffusion coefficients \( D \) with DOSY NMR. Here, I use DOSY NMR to determine whether the species that form upon mixing peptides 1a and 1b are also tetramers. The homotetramers of peptides 1a and 1b have diffusion coefficients of about \( 12 \times 10^{-11} \text{ m}^2/\text{s} \) in D\(_2\)O at 298 K. The diffusion coefficients of the species that predominate in the 1:1 mixture are comparable, \( 11.4 \times 10^{-11} \text{ m}^2/\text{s} \) (Table 3.1), indicating that these species are also tetramers.

**Table 3.1. Diffusion coefficients \( (D) \) of peptides 1a and 1b in D\(_2\)O at 298 K**

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<td>8.0(^b)</td>
<td>11.4 \pm 1.1</td>
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\(^a\)Molecular weight calculated for the neutral (uncharged) peptide.

\(^b\)Total concentration of the 1:1 mixture of peptides 1a and 1b.

In this chapter, I describe the homotetramers and heterotetramers formed by peptides 1a and 1b using the letters A and B. The homotetramers are designated A\(_4\) and B\(_4\), and the 3:1, 2:2, and 1:3 heterotetramers are designated A\(_3\)B\(_1\), A\(_2\)B\(_2\), and A\(_1\)B\(_3\). Two topological isomers of the A\(_2\)B\(_2\) heterotetramer could form: one consisting of two homodimers (A•A and B•B); the other consisting of two heterodimers (A•B and A•B). Figure 3.2 illustrates the homotetramers and heterotetramers, where a single β-strand represents either peptide 1a or 1b.
Figure 3.2. Cartoons illustrating homotetramers and heterotetramers, in which peptide 1a is represented by a blue arrow and peptide 1b is represented by a red arrow.

The complex mixture of monomers, homotetramers, and heterotetramers can give as many as 16 resonances in the $^1$H NMR spectrum: two from the A monomer and A$_4$ homotetramer; two from the B monomer and B$_4$ homotetramer, four from the A$_3$B$_1$ heterotetramer, four from the A$_1$B$_3$ heterotetramer, and either two or four from the A$_2$B$_2$ heterotetramer. The A$_2$B$_2$ heterotetramer would give four resonances if both the A•A/B•B and A•B/A•B topological isomers formed, but only two resonances if just one of the two isomers formed.

Elucidation of the A$_2$B$_2$ Topological Isomer. I used peptides $[^{15}\text{N}]$1a and $[^{15}\text{N}]$1b to elucidate the dimers within the A$_2$B$_2$ heterotetramer. In the preceding chapter, I used these peptides and $^{15}$N-edited NOESY to help establish the pairing of the dimers within the A$_4$ and B$_4$
homotetramers. Here, I compare the $^{15}\text{N}$-edited NOESY spectra of pure $[^{15}\text{N}]1\text{a}$ and pure $[^{15}\text{N}]1\text{b}$ to that of the 1:1 mixture to determine which $A_2B_2$ topological isomer forms (Figure 3.3). The spectra show that the $A_2B_2$ heterotetramer consists of an $A\cdot A$ and a $B\cdot B$ homodimer, and not of two $A\cdot B$ heterodimers (Figure 3.4).
Figure 3.3. $^{15}$N-Edited NOESY spectra of (a) peptide $^{15}$N1a at 8.0 mM, (b) peptide $^{15}$N1b at 8.0 mM, and (c) the 1:1 mixture of peptides $^{15}$N1a and $^{15}$N1b at 8.0 mM total concentration in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K. The G$_{33}$H$_{\alpha}$ corresponds to the pro-$R$ $\alpha$-proton and the G$_{33}$H$_{\alpha}'$ corresponds to the pro-$S$ $\alpha$-proton. Crosspeaks associated with chemical exchange of peptide 1b between the monomer and the B$_4$ and A$_2$B$_2$ tetramers are labeled EX. Dotted lines illustrate how the crosspeaks from the 1:1 mixture compare with the crosspeaks of pure $^{15}$N1a and pure $^{15}$N1b.
Figure 3.4. NOEs involving the $^{15}$NH protons within the A$_2$B$_2$ heterotetramer. (a) The A•A homodimer with blue arrows illustrating the NOEs observed within the dimers. (b) The B•B homodimer with red arrows illustrating the NOEs observed within the dimers. (c) The A•B heterodimer (not formed).
The $^{15}$N-edited NOESY spectrum of the 1:1 mixture of peptides $[^{15}$N]$^1$ and $[^{15}$N]$^1b$ shows four distinct sets of resonances: two sets associated with the A$_2$B$_2$ heterotetramer; one set associated with the A$_4$ homotetramer; and one set associated with the B monomer (Figure 3.3c). In addition to the NOEs, the spectrum also shows crosspeaks associated with chemical exchange between the monomer of peptide $[^{15}$N]$^1b$ and the A$_2$B$_2$ heterotetramer.

The A$_2$B$_2$ heterotetramer gives two sets of resonances: one set from the F$_{20}$NH proton of peptide $[^{15}$N]$^1$ and the other set from the G$_{33}$NH proton of peptide $[^{15}$N]$^1b$. The F$_{20}$NH proton of peptide $[^{15}$N]$^1$ gives a strong interresidue NOE to the F$_{19}$H$\alpha$ proton and a weaker intraresidue NOE to the F$_{20}$H$\alpha$ proton. Figure 3.4a summarizes these NOEs. An intermolecular NOE between the F$_{20}$NH and the A$_{21}$H$\alpha$ protons is not observed as a separate crosspeak because the F$_{20}$H$\alpha$ and the A$_{21}$H$\alpha$ resonances overlap. The F$_{20}$NH proton gives an additional NOE to the A$_{21}$H$\beta$ protons, which corroborates the proximity of these residues (Figure S3.2). An intermolecular NOE is not observed between the F$_{20}$NH proton of peptide $[^{15}$N]$^1$ and the L$_{34}$H$\alpha$ proton of peptide $[^{15}$N]$^1b$ (Figure 3.3c); an intermolecular NOE is also not observed between the F$_{20}$NH proton of peptide $[^{15}$N]$^1$ and the G$_{33}$NH proton of peptide $[^{15}$N]$^1b$ (Figure S3.3). The absence of these two NOEs indicates that peptide $[^{15}$N]$^1$ is not part of an A•B heterodimer (Figure 3.4c).

The G$_{33}$NH proton of peptide $[^{15}$N]$^1b$ gives an interresidue NOE to the I$_{32}$H$\alpha$ proton and intraresidue NOEs to the G$_{33}$H$\alpha$ and G$_{33}$H$\alpha'$ protons (Figure 3.3c). The G$_{33}$NH proton also gives an intermolecular NOE to the L$_{34}$H$\alpha$ proton. This NOE confirms that the B•B homodimer forms within the A$_2$B$_2$ heterotetramer and rules out the A•B heterodimer. Figure 3.4b summarizes these NOEs. Collectively, the $^{15}$N-edited NOESY studies establish that the A•A/B•B topological isomer that forms exclusively is the A$_2$B$_2$ heterotetramer.
\(^1\)H,\(^{15}\)N HSQC Reveals That Peptides \(\[^{15}\)N\]1a and \(\[^{15}\)N\]1b Form Three Heterotetramers: A\(_3\)B\(_1\), A\(_2\)B\(_2\), and A\(_1\)B\(_3\). I compared the \(^1\)H,\(^{15}\)N HSQC spectra of pure \(\[^{15}\)N\]1a and pure \(\[^{15}\)N\]1b to that of the 1:1 mixture to show which crosspeaks are associated with heterotetramers. The \(^1\)H,\(^{15}\)N HSQC spectrum of the 1:1 mixture of peptides \(\[^{15}\)N\]1a and \(\[^{15}\)N\]1b at 8.0 mM total concentration shows 10 new crosspeaks (14 crosspeaks in total). The crosspeaks are sharp and distinct, indicating that the tetramers exchange slowly on the NMR time scale. The two crosspeaks designated 1 and 2 come from the monomer and homotetramer of peptide \(\[^{15}\)N\]1a; the two crosspeaks designated 3 and 4 come from the monomer and homotetramer of peptide \(\[^{15}\)N\]1b. The 10 remaining crosspeaks designated 5–14 come from the heterotetramers. Figure 3.5a shows the \(^1\)H,\(^{15}\)N HSQC spectrum of the 1:1 mixture of peptides \(\[^{15}\)N\]1a and \(\[^{15}\)N\]1b. Table 3.2 summarizes the chemical shifts of crosspeaks 1–14.
Figure 3.5. $^1$H, $^{15}$N HSQC spectra of 8.0 mM mixtures in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K of peptides: (a) $[^{15}$N]$^1$a and $[^{15}$N]$^1$b; (b) $[^{15}$N]$^1$a and $^1$b; (c) $^1$a and $[^{15}$N]$^1$b. The asterisk (*) indicates a crosspeak from a minor unidentified species associated with peptide $[^{15}$N]$^1$b.
Table 3.2. Chemical shifts of peptides $[^{15}\text{N}]1\text{a}$ and $[^{15}\text{N}]1\text{b}$

<table>
<thead>
<tr>
<th>crosspeak</th>
<th>$\delta F_{20}$</th>
<th>$\delta G_{33}$</th>
<th>species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.32</td>
<td>122.3</td>
<td>A monomer</td>
</tr>
<tr>
<td>2</td>
<td>8.56</td>
<td>121.3</td>
<td>$A_4$ homotetramer</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>8.39</td>
<td>$B_1$ monomer</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>9.33</td>
<td>$B_2$ homotetramer</td>
</tr>
<tr>
<td>5</td>
<td>8.81</td>
<td>124.9</td>
<td>$A_2B_2$ heterotetramer</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>9.03</td>
<td>$A_2B_2$ heterotetramer</td>
</tr>
<tr>
<td>7</td>
<td>8.69</td>
<td>125.7</td>
<td>$A_2B_2$ heterotetramer</td>
</tr>
<tr>
<td>8</td>
<td>8.66</td>
<td>121.1</td>
<td>$A_2B_2$ heterotetramer</td>
</tr>
<tr>
<td>9</td>
<td>8.60</td>
<td>119.3</td>
<td>$A_2B_2$ heterotetramer</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>7.94</td>
<td>$A_2B_2$ heterotetramer</td>
</tr>
<tr>
<td>11</td>
<td>8.92</td>
<td>120.9</td>
<td>$A_2B_2$ heterotetramer</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>8.74</td>
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</tr>
<tr>
<td>13</td>
<td>-</td>
<td>9.25</td>
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</tr>
<tr>
<td>14</td>
<td>-</td>
<td>9.17</td>
<td>$A_2B_2$ heterotetramer</td>
</tr>
</tbody>
</table>

$[^{1}]H,^{15}\text{N}$ HSQC spectrum was recorded for the 1:1 mixture at 8.0 mM in 9:1 H$_2$O/D$_2$O at 293 K.

The remaining crosspeaks 5–14 come from the $A_3B_1$, $A_2B_2$, and $A_1B_3$ heterotetramers. Crosspeaks 5 and 6 are prominent and strikingly similar in intensity to each other. These two crosspeaks come from the $A_2B_2$ heterotetramer. Crosspeaks 7–14 are weaker and are also similar in intensity to each other. These eight crosspeaks are associated with the $A_3B_1$ and $A_1B_3$ heterotetramers.

I mixed peptides $[^{15}\text{N}]1\text{a}$ and $1\text{b}$ and also mixed peptides $1\text{a}$ and $[^{15}\text{N}]1\text{b}$ to assign crosspeaks 7–14 to the respective peptides. Figures 3.5b and 3.5c show the $^1\text{H},^{15}\text{N}$ HSQC spectra of these mixtures of labeled and unlabeled peptides. The $^1\text{H},^{15}\text{N}$ HSQC spectrum of peptides $[^{15}\text{N}]1\text{a}$ and $1\text{b}$ shows that crosspeaks 1, 2, 5, 7, 8, 9, and 11 come from peptide $[^{15}\text{N}]1\text{a}$; the $^1\text{H},^{15}\text{N}$ HSQC spectrum of peptides $1\text{a}$ and $[^{15}\text{N}]1\text{b}$ shows that crosspeaks 3, 4, 6, 10, 12, 13, and 14 come from peptide $[^{15}\text{N}]1\text{b}$. These spectra confirm that half of the crosspeaks come from peptide $1\text{a}$ and that half of the crosspeaks come from peptide $1\text{b}$.

Assigning the $^1\text{H},^{15}\text{N}$ HSQC Crosspeaks of the $A_3B_1$ and $A_1B_3$ Heterotetramers. To assign which of the crosspeaks 7–14 come from the $A_3B_1$ heterotetramer and which come from the $A_1B_3$ heterotetramer, I compared $^1\text{H},^{15}\text{N}$ HSQC spectra of 3:1 and 1:3 mixtures of peptides $[^{15}\text{N}]1\text{a}$ and $[^{15}\text{N}]1\text{b}$ to that of the 1:1 mixture. In the spectra of the 3:1, 1:1, and 1:3 mixtures,
the relative intensities of crosspeaks 1–14 vary, but the chemical shifts do not. The \( f_1 \) projections of the \(^1\text{H},15\text{N} \) HSQC spectra conveniently illustrate the relative intensities of the crosspeaks as one-dimensional \(^{15}\text{N} \) spectra. Figure 3.6 shows the \( f_1 \) projections of pure \([^{15}\text{N}]1\text{a}\), the 3:1, 1:1, and 1:3 mixtures, and pure \([^{15}\text{N}]1\text{b}\).

![Figure 3.6](image)

**Figure 3.6.** \(^{15}\text{N} \) spectra from the \( f_1 \) projections of the \(^1\text{H},^{15}\text{N} \) HSQC spectra of mixtures of peptides \([^{15}\text{N}]1\text{a}\) and \([^{15}\text{N}]1\text{b}\). Spectra were recorded at 8.0 mM total concentration and varying mole fractions of peptide in 9:1 H\(_2\)O/D\(_2\)O at 600 MHz and 293 K. The mole fraction of peptide \([^{15}\text{N}]1\text{b}\) is designated \( \chi_B \).

The systematic variation of the crosspeaks as a function of the mole fraction \( \chi_B \) clearly establishes which crosspeaks are associated with the A\(_3\)B\(_1\) heterotetramer and which are associated with the A\(_1\)B\(_3\) heterotetramer. Crosspeaks 7–10 have maximum relative intensities at
\( \chi_B = 0.25 \) and come from the A_3B_1 heterotetramer. Crosspeaks 11–14 have maximum relative intensities at \( \chi_B = 0.75 \) and come from the A_1B_3 heterotetramer. Figure 3.7 illustrates relative integrations of the crosspeaks versus the mole fraction of peptide \([^{15}\text{N}]1\text{b}, \chi_B\).

**Figure 3.7.** Plot of the relative integrations of crosspeaks 1–14 versus the mole fraction of peptide \([^{15}\text{N}]1\text{b}, \chi_B\). The intensities were measured by integrating the crosspeaks in the \(^1\text{H},^{15}\text{N}\) HSQC spectra of the mixtures of peptides \([^{15}\text{N}]1\text{a} \) and \([^{15}\text{N}]1\text{b}\).

**Job’s Method of Continuous Variation.** I used Job’s method to determine the relative stabilities of the homotetramers and the heterotetramers of peptides \([^{15}\text{N}]1\text{a} \) and \([^{15}\text{N}]1\text{b}\). Although this method was first introduced to study inorganic complexes, it is useful in all areas of chemistry for studying molecular association.\(^{14,15}\) Job’s method is performed by mixing two compounds “A” and “B” in varying ratios while keeping the total concentration constant. The amount of a complex that forms is then plotted versus the mole fraction to give a plot known as a “Job plot”. The appearance of the Job plot reflects the stoichiometry and relative stability of each complex. The mole fraction at which the maximum amount of the complex forms corresponds
with its stoichiometry. For example, an $A_1B_2$ heterotrimer would give a maximum in a 1:2 mixture ($\chi_B = 0.67$).

I applied Job’s method to peptides $[^{15}\text{N}]\text{Ia}$ and $[^{15}\text{N}]\text{Ib}$, recording $^1\text{H},^{15}\text{N}$ HSQC spectra for nine samples at 8.0 mM total concentration. I plotted the sum of the relative integrals of the $^1\text{H},^{15}\text{N}$ HSQC crosspeaks for each species versus the mole fraction of peptide $[^{15}\text{N}]\text{Ib}$, $\chi_B$. For example, I plotted the curve for the $A_3B_1$ heterotetramer species using the sum of the relative integrals of crosspeaks 7–10. Figure 3.8 illustrates the resulting Job plot.

![Figure 3.8](image)

**Figure 3.8.** Job plot for peptides $[^{15}\text{N}]\text{Ia}$ and $[^{13}\text{N}]\text{Ib}$ showing the relative integrations of the monomers, homotetramers, and heterotetramers versus the mole fraction of peptide $[^{15}\text{N}]\text{Ib}$, $\chi_B$. The curves reflect a monomer-tetramer equilibrium model fitted to the data. The error bars reflect the standard deviations among the individual measurements used to determine the relative integrations of $A_3B_1$, $A_2B_2$, and $A_1B_3$. The relative stabilities determined for each species are $\phi_{44} = 1.00$, $\phi_{43} = 0.22$, $\phi_{42} = 0.67$, $\phi_{41} = 0.12$, $\phi_{40} = 0.12$, $\phi_{11} = 0.36$, and $\phi_{10} = 2.20$.

The Job plot shows that the $A_2B_2$ heterotetramer predominates over a wide range of mole fractions. At low mole fractions, $\chi_B \leq 0.25$, the $A_4$ homotetramer predominates. At high mole fractions, $\chi_B \geq 0.75$, the $B$ monomer and $B_4$ heterotetramer predominate. The $A_2B_2$ heterotetramer reaches a maximum concentration at a mole fraction $\chi_B$ slightly greater than 0.50.
The $A_3B_1$ heterotetramer and the $A_1B_3$ heterotetramer form to a lesser extent, reaching a maximum concentration at low and high mole fractions $\chi_B$, respectively.

**Simulated Job Plots of Homotetramers and Heterotetramers.** I generated simulated Job plots reflecting different homotetramer and heterotetramer stabilities to help interpret the data in Figure 3.8. I used an implementation developed by Collum and coworkers that readily accommodates homotetramer and heterotetramer equilibria. I simulated a Job plot for a statistical distribution of homotetramers and heterotetramers and Job plots in which one of the heterotetramers is favored. These plots demonstrate how the relative stabilities of the tetramers affect the shapes of the curves. Figure 3.9 illustrates the resulting Job plots; the relative integrations of the species are plotted versus the mole fraction $\chi_B$. 
Figure 3.9. Simulated Job plots that show the relative integrations of the monomers, homotetramers, and heterotetramers versus the mole fraction of B, $\chi_B$. (a) A statistical distribution of homotetramers and heterotetramers; $\phi_{44} = \phi_{43} = \phi_{42} = \phi_{41} = \phi_{40} = 1$. (b) $A_2B_2$ heterotetramer is favored; $\phi_{42} = 2$ and $\phi_{43} = \phi_{44} = \phi_{41} = \phi_{40} = 1$. (c) $A_3B_1$ heterotetramer is favored; $\phi_{43} = 2$ and $\phi_{44} = \phi_{42} = \phi_{41} = \phi_{40} = 1$. (d) $A_1B_3$ heterotetramer is favored; $\phi_{41} = 2$ and $\phi_{43} = \phi_{44} = \phi_{42} = \phi_{40} = 1$. (e) A statistical distribution of homotetramers and heterotetramers that also includes monomers; $\phi_{43} = \phi_{44} = \phi_{42} = \phi_{41} = \phi_{40} = 1$ and $\phi_{10} = 1$. (f) A statistical distribution of homotetramers and heterotetramers that also includes monomers, where the B monomer is favored $\phi_{44} = \phi_{43} = \phi_{42} = \phi_{41} = \phi_{40} = 1, \phi_{11} = 1,$ and $\phi_{10} = 2$.

In the implementation by Collum and coworkers, the relative concentrations of the homotetramers and heterotetramers are calculated from equations based on a homotetramer-heterotetramer equilibrium model. The parameters $\phi_{Nn}$ are ascribed to each of the homotetramers and heterotetramers in the equations, where the subscripts $N$ and $n$ are integers in which the value of $N$ describes the oligomer size and the value of $n$ describes the number of "A" subunits. The value of each $\phi_{Nn}$ reflects the relative stability of each homotetramer or heterotetramer. The
parameters $\phi_{44}$, $\phi_{43}$, $\phi_{42}$, $\phi_{41}$, and $\phi_{40}$ describe the relative stabilities of $A_4$, $A_3B_1$, $A_2B_2$, $A_1B_3$, and $B_4$, respectively. When each tetramer is equally stable, all parameters are equal (e.g., $\phi_{44} = \phi_{43} = \phi_{42} = \phi_{41} = \phi_{40} = 1$) and a statistical distribution of homotetramers and heterotetramers forms.

The Job plot of a statistical distribution of homotetramers and heterotetramers is symmetrical, where the maximum of each curve reflects the tetramer stoichiometry. In the 1:1 mixture, the $A_2B_2$ heterotetramer predominates, with smaller fractions of the $A_3B_1$ and $A_1B_3$ heterotetramers in equal amounts, and with traces of the $A_4$ and $B_4$ homotetramers in equal amounts. In the 3:1 mixture, the $A_3B_1$ heterotetramer predominates, with smaller fractions of the $A_4$ homotetramer and $A_2B_2$ heterotetramer, and with traces of the $A_1B_3$ heterotetramer. Similarly, in the 1:3 mixture, the $A_1B_3$ heterotetramer predominates, with smaller fractions of the $B_4$ homotetramer and $A_2B_2$ heterotetramer, and with traces of the $A_3B_1$ heterotetramer. Figure 3.9a illustrates the Job plot for a statistical distribution of homotetramers and heterotetramers.

The appearance of the Job plot changes if any of the tetramers are favored or disfavored. If the $A_2B_2$ tetramer is favored, the $A_2B_2$ curve shows a pronounced increase and the $A_3B_1$ and $A_1B_3$ curves diminish slightly (Figure 3.9b). If the $A_3B_1$ tetramer is favored, the $A_3B_1$ curve shows a pronounced increase and the $A_2B_2$ curve diminishes slightly (Figure 3.9c). If the $A_1B_3$ tetramer is favored, the $A_1B_3$ curve shows a pronounced increase and the $A_2B_2$ curve diminishes slightly (Figure 3.9d).

**Analysis of the Job Plot.** I modified the implementation by Collum and coworkers to accommodate the equilibrium of the monomers with the homotetramers and heterotetramers. In our implementation, the relative concentrations of the monomers, homotetramers, and heterotetramers are calculated from equations based on a monomer-homotetramer-heterotetramer equilibrium model. The parameters $\phi_{11}$ and $\phi_{10}$ reflect the relative stabilities of the monomers $A$
and B. The Job plot of a statistical distribution of homotetramers and heterotetramers that also includes the monomers is similar to the Job plot without monomers, except that the fraction of each tetramer is slightly diminished (Figure 3.9e). If the equilibrium favors one of the two monomers, a greater fraction of that monomer forms (Figure 3.9f).

I analyzed the data from our Job’s method experiment by nonlinear least-squares fitting of the model to the data. During the fit, the parameters $\phi_{43}$, $\phi_{42}$, $\phi_{41}$, $\phi_{40}$, $\phi_{11}$, and $\phi_{10}$ were allowed to vary, while the parameter $\phi_{44}$ remained fixed at 1. Figure 3.8 illustrates the Job plot with the fitted curves ($\phi_{44} = 1.00$, $\phi_{43} = 0.22$, $\phi_{42} = 0.67$, $\phi_{41} = 0.12$, $\phi_{40} = 0.12$, $\phi_{11} = 0.36$, $\phi_{10} = 2.20$).

The model fits the data well. The quality of the fit corroborates that peptides $[^{15}\text{N}]1\text{a}$ and $[^{15}\text{N}]1\text{b}$ form a mixture of homotetramers and heterotetramers. The appearance of the resulting plot does not resemble the statistical distribution shown in Figure 3.9e. The Job plot shows little or no preference for the A2B2 heterotetramer, but it does show suppression of the A3B1 and A1B3 heterotetramers.

The Job’s method of continuous variation study and nonlinear least-squares fitting of the data establish that peptides 1a and 1b prefer to segregate within the heterotetramers. The suppression of the A3B1 and A1B3 heterotetramers shows that the A•B heterodimer subunit is disfavored and that heterotetramers containing an A•B heterodimer subunit are less stable. This finding explains why the A2B2 heterotetramer contains two homodimers rather than two heterodimers. Peptide 1a, which contains Aβ17–23, prefers to pair with itself to form a hydrogen-bonded homodimer; peptide 1b, which contains Aβ30–36, prefers to pair with itself to form a hydrogen-bonded homodimer.

**Molecular Models of A2B2 Heterotetramers.** I constructed energy-minimized models of A2B2 heterotetramers to help understand the preferential pairing of peptides 1a and 1b to form
homodimers. By combining the monomer subunits of the models of the A₄ and B₄ homotetramers developed in the preceding chapter⁶ and re-minimizing, I generated two models of the A₂B₂ heterotetramers: the A•A/B•B topological isomer that was observed, and the A•B/A•B topological isomer that was not. Figure 3.10 illustrates the resulting models of these two topological isomers.

![Molecular models of the topological isomers of the A₂B₂ heterotetramer of peptides 1a and 1b.](image)

Figure 3.10. Molecular models of the topological isomers of the A₂B₂ heterotetramer of peptides 1a and 1b. (a) The A•A/B•B topological isomer. (b) The A•B/A•B topological isomer. Each model is a minimum-energy structure (local minimum) generated with MacroModel using the MMFFs force field with GB/SA water solvation.
The models show that the A2B2 heterotetramers can form sandwich-like structures that are similar to the homotetramers. Both topological isomers consist of two, four-stranded β-sheets that laminate together through hydrophobic packing. The side chains of L17, F19, and A21 from peptide 1a and of A30, I32, L34, and V36 from peptide 1b form hydrophobic surfaces that pack in the hydrophobic core of each heterotetramer. The interface between the A•A and B•B homodimers in the A•A/B•B topological isomer is uniformly packed. In contrast, the interface between the two A•B heterodimers in the A•B/A•B topological isomer is densely packed at one end and lightly packed at the other (Figure 3.10b).

The A•A homodimer of peptide 1a exhibits a large hydrophobic surface, with intimate contacts between the side chains of L17, F19, and A21. The large F19 and small A21 residues fit together well to help provide a uniformly packed surface (Figure 3.11a). The B•B homodimer of peptide 1b also exhibits a large hydrophobic surface, with intimate contacts between the side chains of A30, I32, L34, and V36. These residues also provide a uniformly packed surface (Figure 3.11b). The A•B heterodimer exhibits a hydrophobic surface with intimate contacts between the side chains of L17, F19, and A21 from peptide 1a and the side chains of I32, L34, and V36 from peptide 1b. The side chains do not pack uniformly, but rather the side chains pack densely at one end of the dimer and pack lightly at the other (Figure 3.11c).
Figure 3.11. Molecular models of the homodimer and heterodimer subunits of the A₂B₂ heterotetramers of peptides 1a and 1b.

These molecular models suggest that differences between the homodimers and heterodimers formed by peptides 1a and 1b dictate the observed differences in the A₂B₂
heterotetramer stability. The uniform packing of the A•A and B•B homodimers appears to drive the formation of the observed A₂B₂ heterotetramer. The non-uniform packing of the A•B heterodimer appears to suppress the formation of the A₃B₁ and A₁B₃ heterotetramers, and also the alternative topological isomer of the A₂B₂ heterotetramer.

CONCLUSION

In framing the question behind these studies, I set out to determine whether peptides derived from the central and C-terminal regions of Aβ prefer to coassemble or to segregate. I found that the answer is more nuanced, at least in the context of the model system provided by peptides 1. Peptides 1a and 1b can coassemble, but the resulting heterotetramers reflect a preference to segregate within the dimer subunits. The heterotetramers comprising heterodimers are disfavored, while the heterotetramers comprising homodimers are not. These findings recapitulate the segregation within Aβ₁₋₄₀ fibrils, in which the central region assembles to form a hydrogen-bonded β-sheet and the C-terminal region assembles to form a hydrogen-bonded β-sheet. The two β-sheets coassemble through hydrophobic contacts.

¹⁵N-Isotopic labeling, ¹H,¹⁵N NMR spectroscopy, and Job’s method of continuous variation proved essential in these studies. Incorporation of a single ¹⁵N-isotopic label provided a sensitive and non-perturbing spectroscopic probe. ¹⁵N-Labeled peptides are readily prepared from commercially available ¹⁵N-labeled amino acids using solid-phase peptide synthesis. ¹H,¹⁵N HSQC facilitated identification of the monomers, homotetramers, and heterotetramers. Job’s method of continuous variation assigned the resonances of each monomer and tetramer and established the relative stability of the tetramers. ¹⁵N-Edited NOESY established the identity of the topological isomer of the A₂B₂ heterotetramer.
These techniques, which proved useful for elucidating the assembly and coassembly of β-sheet peptides, should also be valuable in broader contexts. Peptide and protein assemblies occur widely in coiled coils, helix bundles, and collagen helices, as well as in amyloid oligomers and other β-sheet supramolecular assemblies. I envision that $^{15}\text{N}$-isotopic labeling in conjunction with $^{1}\text{H}$, $^{15}\text{N}$ NMR spectroscopy and Job’s method will also be valuable for studying these assemblies.
REFERENCES AND NOTES


12. I used $^1$H NMR TOCSY to assign residues associated with these NOEs (Figures 3.S1 and 3.S2).

13. The $^1$H NMR NOESY spectrum of the 1:1 mixture of peptides 1a and 1b shows additional NOEs associated with the stacking of the two homodimers to form a sandwich-like tetramer. The spectrum shows NOEs between the F$_{19}$ aromatic protons of peptide 1a and the I$_{32}$ and L$_{34}$ side-chain protons of peptide 1b (Figure S3.4a). The spectrum also shows NOEs between the A$_{21}$ side-chain protons of peptide 1a and the I$_{32}$ side-chain protons of peptide 1b (Figure S3.4b). Figure S3.5 illustrates the stacking of the A•A and B•B homodimers of peptides 1a and 1b consistent with these NOEs.


16. The 8.0 mM total concentration was chosen to favor tetramers in the mixtures of peptides $[^{15}\text{N}]\text{1a}$ and $[^{15}\text{N}]\text{1b}$.


Coassembly of Peptides Derived from β-Sheet Regions of

β-Amyloid
3.1. **Supplemental Figures**

![Figure S3.1](image)

**Figure S3.1.** $^{15}$N-Edited TOCSY spectrum of the 1:1 mixture of peptides $[^{15}\text{N}]\text{Ia}$ and $[^{15}\text{N}]\text{Ib}$ at 8.0 mM total concentration in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K.
Figure S3.2. Comparison of the (a) $^{15}$N-Edited NOESY spectrum of the 1:1 mixture of peptides $^{[15N]}1a$ and $^{[15N]}1b$ with the (b) $^1$H NMR TOCSY spectrum of the 1:1 mixture of peptides 1a and 1b in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K. Key crosspeaks associated with the A$_2$B$_2$ heterotetramer are highlighted in red from F$_{19}$, F$_{20}$, and A$_{21}$ of peptides $^{[15N]}1a$ and 1a, and also from I$_{32}$, G$_{33}$, L$_{34}$ of peptides $^{[15N]}1b$ and 1b.
Figure S3. $^{15}$N-edited NOESY spectrum of the 1:1 mixture of peptides [${}^{15}$N]1a and [${}^{15}$N]1b at 8.0 mM total concentration in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K. Crosspeaks associated with chemical exchange between the monomers and tetramers are labeled EX. The asterisk (*) indicates a crosspeak from a minor unidentified species associated with the A$_2$B$_2$ heterotetramer.
Figure S3.4. Expansions of the $^1$H NMR NOESY spectrum of the 1:1 mixture of peptides 1a and 1b at 8.0 mM total concentration in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K. Key interlayer NOEs between A·A and B·B homodimers from the A$_2$B$_2$ heterotetramer are highlighted in red. $F_{19}H_0$, $F_{19}H_m$, and $F_{19}H_p$, correspond to the ortho, meta, and para protons of $F_{19}$. 
Figure S3.5. $A_2B_2$ heterotetramer consisting of two hydrogen-bonded homodimers of peptides 1a and 1b. Contacts between the side chains of F$_{19}$ and L$_{34}$ and between the side chains of A$_{21}$ and I$_{32}$ are shown, reflecting observed NOEs.
3.II. M ATERIALS AND METHODS

Synthesis of Peptides 1

Synthesis and purification of peptides 1a and 1b, and \([^{15}\text{N}]1\text{a}\) and \([^{15}\text{N}]1\text{b}\) were performed as described in the preceding chapter.\(^1\)

Fmoc-Protection of \(^{15}\text{N}-\text{Labeled Amino Acids}\)

Fmoc-protection of \(^{15}\text{N}\)-labeled glycine and phenylalanine was performed as described in the preceding chapter.\(^1,2\)

NMR Spectroscopy of Peptides 1

Sample Preparation. NMR spectroscopy of peptides 1a and 1b was performed in D\(_2\)O (D, 99.96%; Cambridge Isotope Laboratories, Inc.). The solutions were prepared by dissolving a weighed portion of the peptide in the appropriate volume of solvent. The molecular weights of the peptides were calculated as the TFA salts with all amino groups assumed to be protonated (1a, M.W. 2223.85 g/mol and 1b, M.W. 2099.91 g/mol). The solutions were allowed to stand for 24 h to allow complete hydrogen to deuterium exchange of the amide NH protons.

\(^1\)H NMR, TOCSY, and NOESY Data Collection. NMR spectra were recorded on a Bruker 600 MHz spectrometer with a TBI probe. Presaturation water suppression was applied as needed. TOCSY spectra were recorded with 2048 points in the \(f_2\) dimension and 512 increments in the \(f_1\) dimension with a 150-ms spin-lock mixing time. NOESY spectra were recorded with 2048 points in the \(f_2\) dimension and 512 increments in the \(f_1\) dimension with a 150-ms mixing time.
$^1$H NMR, TOCSY, and NOESY Data Processing. NMR spectra were processed with Bruker XwinNMR software. Automatic baseline correction was applied in both dimensions after phasing the spectra. TOCSY spectra were Fourier transformed to a final matrix size of 2048 x 2048 real points using a Qsinc weighting function (GB = 0.05) and forward linear prediction. NOESY spectra were Fourier transformed to a final matrix size of 2048 x 2048 real points using a Qsinc weighting function (GB = 0.05) and forward linear prediction.

Diffusion-Ordered Spectroscopy (DOSY) Experiments. DOSY experiments were performed on a Bruker 500 MHz spectrometer equipped with a TCI cryoprobe, with a diffusion delay ($\Delta$) of 75-ms and a diffusion gradient length ($\delta$) of 2.5-ms. Sixteen sets of FIDs were recorded with the gradient strength incremented from 5%–95% using a linear ramp. The combined FIDs were Fourier transformed in Bruker's TopSpin™ software to give a pseudo-2D spectrum. After phasing and performing baseline correction, each pseudo-2D spectrum was processed with logarithmic scaling on the Y-axis. The Y-axis was calibrated to the diffusion coefficient of the residual HOD peak in D$_2$O (1.9 x 10$^{-9}$ m$^2$/s at 298 K). The diffusion coefficients of the peptides were read and converted from logarithmic values to linear values.

NMR Spectroscopy of Peptides $[^{15}$N]$1$

Sample Preparation. NMR spectroscopy of peptides $[^{15}$N]$1a$ and $[^{15}$N]$1b$ was performed in 9:1 H$_2$O/D$_2$O. The solutions were prepared by dissolving a weighed portion of the peptide in the appropriate volume of solvent. The molecular weights of the peptides were calculated as the TFA salts with all amino groups assumed to be protonated ($[^{15}$N]$1a$, M.W. 2224.85 g/mol and
[\textsuperscript{15}N]1b, M.W. 2100.91 g/mol. 4,4-Dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA) was added as an internal standard for referencing chemical shifts.\textsuperscript{4}

\textsuperscript{1}H NMR, \textsuperscript{1}H,\textsuperscript{15}N HSQC, \textsuperscript{1}H,\textsuperscript{15}N TOCSY-HSQC (\textsuperscript{15}N-edited TOCSY), and \textsuperscript{1}H,\textsuperscript{15}N NOESY-HSQC (\textsuperscript{15}N-edited NOESY) Data Collection. NMR spectra were recorded on a Bruker 600 MHz spectrometer with either a TBI probe or a BBFO cryoprobe. Gradient water suppression was applied as needed. \textsuperscript{1}H,\textsuperscript{15}N HSQC spectra were recorded with 1024 points in the \( f_2 \) dimension and 512 increments in the \( f_1 \) dimension. \textsuperscript{1}H,\textsuperscript{15}N TOCSY-HSQC spectra were recorded with a 150-ms spin-lock mixing time, and with 2048 points in the \( f_3 \) dimension (\textsuperscript{1}H), one increment in the \( f_2 \) dimension (\textsuperscript{15}N), and 512 increments in the \( f_1 \) dimension (\textsuperscript{1}H). \textsuperscript{1}H,\textsuperscript{15}N NOESY-HSQC spectra were recorded with a 150-ms mixing time, and with 2048 points in the \( f_3 \) dimension (\textsuperscript{1}H), 1 increment in the \( f_2 \) dimension (\textsuperscript{15}N), and 1024 increments in the \( f_1 \) dimension (\textsuperscript{1}H).

\textsuperscript{1}H NMR, \textsuperscript{1}H,\textsuperscript{15}N HSQC, \textsuperscript{1}H,\textsuperscript{15}N TOCSY-HSQC (\textsuperscript{15}N-edited TOCSY), and \textsuperscript{1}H,\textsuperscript{15}N NOESY-HSQC (\textsuperscript{15}N-edited NOESY) Data Processing. NMR spectra were Fourier transformed in Bruker XwinNMR software with forward linear prediction and a Qsinc weighting function. Automatic baseline correction was applied in both dimensions after phasing the spectra. The \textsuperscript{1}H,\textsuperscript{15}N HSQC spectra were processed to a final matrix size of 2048 x 1024 real points and with GB = 0.1 in the \( f_2 \) dimension. The \textsuperscript{1}H,\textsuperscript{15}N TOCSY-HSQC spectra were processed to a final 2D matrix size of 2048 x 1024 real points (\( f_3, f_1 \)) and with GB = 0.05 in both dimensions. The \textsuperscript{1}H,\textsuperscript{15}N NOESY-HSQC spectra were processed to a final 2D matrix size of 4096 x 2048 real points (\( f_3, f_1 \)) and with GB = 0.05 in both dimensions.
Molecular Modeling of Peptides 1a and 1b.

Molecular models of the A₂B₂ heterotetramers were generated using the models and methods from the preceding chapter.¹ The A₄ and B₄ homotetramers of peptides 1a and 1b were imported into PyMOL: Peptide monomers were selected to construct the A·A and B·B homodimer subunits within the A·A/B·B topological isomer. Peptide monomers were selected to construct the two A·B heterodimer subunits within the A·B/A·B topological isomer. The dimer subunits were oriented so that the side chains of L₁₇, F₁₉, A₂₁, and D₂₃ and the side chains of A₃₀, I₃₂, L₃₄, and V₃₆ formed the hydrophobic core of the A₂B₂ heterotetramers.

The coordinates were exported from PyMOL as a .pdb file. The file was imported into MacroModel with the Maestro user interface. Atom types and bond orders were edited as needed to correct errors in bond type and charge. Distance constraints were applied to reflect the folding and dimerization of the macrocycles. Four interlayer distance constraints between the δ-methyl group of Ile₁₁ and the methoxy group of Hao were applied to reflect the observed interlayer contacts. Minimization was performed with the MMFFs force field and GB/SA water solvation. All constraints were removed and minimization was repeated to generate a minimum-energy conformation (local minimum). The coordinates were exported in .pdb file format and imported into PyMOL.
Job’s Method of Continuous Variation

Nine samples of peptides $[^{15}\text{N}]1\text{a}$ and $[^{15}\text{N}]1\text{b}$ were prepared at 8.0 mM total concentration with mole fractions of peptide $[^{15}\text{N}]1\text{b} = 0.00, 0.125, 0.25, 0.375, 0.50, 0.625, 0.75, 0.875, and 1.00. An $^1\text{H},^{15}\text{N}$ HSQC spectrum at 600 MHz and 293 K was recorded for each mixture using the data collection and data processing parameters described above. These spectra are shown on pages S40-48.

The spectra were reprocessed in Bruker’s TopSpin™ software using a Qsine weighting function to sharpen the crosspeaks for measuring the intensities. One-dimensional $^{15}\text{N}$ spectra from the two-dimensional $^1\text{H},^{15}\text{N}$ HSQC spectra were generated by typing “f1sum” in the command line. A stack plot of the $^{15}\text{N}$ spectra is shown on page S49.

The volume integrals of the crosspeaks in the $^1\text{H},^{15}\text{N}$ HSQC spectra were measured and normalized to 1.0. Table S3.1 summarizes the volume integrals versus the mole fraction of peptide $[^{15}\text{N}]1\text{b}, \chi_B$.

**Table S3.1. Relative integrals of the crosspeaks $I–14$ from the $^1\text{H},^{15}\text{N}$ HSQC spectra**

| $\chi_B$ | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 |
|----------|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 0.000    | 0.0664 | 0.9336 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 0.125    | 0.0449 | 0.6883 | 0.0608 | 0.0000 | 0.0438 | 0.0435 | 0.0363 | 0.0268 | 0.0427 | 0.0125 | 0.0000 | 0.0000 | 0.0000 |
| 0.250    | 0.0332 | 0.4359 | 0.1089 | 0.0005 | 0.1309 | 0.1345 | 0.0255 | 0.0421 | 0.0318 | 0.0466 | 0.0031 | 0.0030 | 0.0028 | 0.0012 |
| 0.375    | 0.0281 | 0.2986 | 0.1445 | 0.0042 | 0.1854 | 0.1785 | 0.0424 | 0.0393 | 0.0335 | 0.0239 | 0.0039 | 0.0076 | 0.0064 | 0.0037 |
| 0.500    | 0.0177 | 0.1350 | 0.1987 | 0.0209 | 0.2330 | 0.2310 | 0.0205 | 0.0308 | 0.0262 | 0.0281 | 0.0195 | 0.0158 | 0.0087 | 0.0140 |
| 0.625    | 0.0115 | 0.0608 | 0.2699 | 0.0384 | 0.2423 | 0.2325 | 0.0207 | 0.0206 | 0.0148 | 0.0134 | 0.0278 | 0.0224 | 0.0216 | 0.0033 |
| 0.750    | 0.0082 | 0.0096 | 0.3095 | 0.1466 | 0.1782 | 0.1734 | 0.0037 | 0.0097 | 0.0071 | 0.0055 | 0.0435 | 0.0390 | 0.0293 | 0.0368 |
| 0.875    | 0.0044 | 0.0009 | 0.3820 | 0.2741 | 0.0812 | 0.0783 | 0.0035 | 0.0020 | 0.0000 | 0.0006 | 0.0466 | 0.0351 | 0.0475 | 0.0440 |
| 1.000    | 0.0000 | 0.0000 | 0.4796 | 0.5204 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
To generate the Job plot, the relative integrations of the monomers, homotetramers, and heterotetramers were plotted versus the mole fraction $\chi_B$. The normalized integrals of crosspeaks 1 and 2 were used for the relative integrations of the A monomer and A$_4$ homotetramer, respectively; the normalized integrals of crosspeaks 3 and 4 were used for the relative integrations of the B monomer and B$_4$ homotetramer, respectively. The sum of the normalized integrals of crosspeaks 5 and 6 was used for the relative integration of the A$_2$B$_2$ heterotetramer; the sum of the normalized integrals of crosspeaks 7–10 was used for the relative integration of the A$_3$B$_1$ heterotetramer; and the sum of the normalized integrals of crosspeaks 11–14 was used for the relative integration of the A$_1$B$_3$ heterotetramer. Table S3.2 summarizes the relative integrations. Figure 8 illustrates the resulting Job plot.

Table S3.2. Relative integrations for the monomers, homotetramers, and heterotetramers

<table>
<thead>
<tr>
<th>$\chi_B$</th>
<th>A</th>
<th>B</th>
<th>A$_4$</th>
<th>A$_3$B$_1$</th>
<th>A$_2$B$_2$</th>
<th>A$_1$B$_3$</th>
<th>B$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.0664</td>
<td>0.0000</td>
<td>0.9336</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>0.125</td>
<td>0.0449</td>
<td>0.0608</td>
<td>0.6883</td>
<td>0.1183</td>
<td>0.0873</td>
<td>0.0003</td>
<td>0.0000</td>
</tr>
<tr>
<td>0.250</td>
<td>0.0332</td>
<td>0.1089</td>
<td>0.4359</td>
<td>0.1459</td>
<td>0.2654</td>
<td>0.0102</td>
<td>0.0005</td>
</tr>
<tr>
<td>0.375</td>
<td>0.0281</td>
<td>0.1445</td>
<td>0.2986</td>
<td>0.1391</td>
<td>0.3639</td>
<td>0.0216</td>
<td>0.0042</td>
</tr>
<tr>
<td>0.500</td>
<td>0.0177</td>
<td>0.1987</td>
<td>0.1350</td>
<td>0.1057</td>
<td>0.4640</td>
<td>0.0581</td>
<td>0.0209</td>
</tr>
<tr>
<td>0.625</td>
<td>0.0115</td>
<td>0.2699</td>
<td>0.0608</td>
<td>0.0695</td>
<td>0.4748</td>
<td>0.0751</td>
<td>0.0384</td>
</tr>
<tr>
<td>0.750</td>
<td>0.0082</td>
<td>0.3095</td>
<td>0.0096</td>
<td>0.0260</td>
<td>0.3516</td>
<td>0.1486</td>
<td>0.1466</td>
</tr>
<tr>
<td>0.875</td>
<td>0.0044</td>
<td>0.3820</td>
<td>0.0009</td>
<td>0.0060</td>
<td>0.1595</td>
<td>0.1731</td>
<td>0.2741</td>
</tr>
<tr>
<td>1.000</td>
<td>0.0000</td>
<td>0.4796</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.5204</td>
</tr>
</tbody>
</table>
3.III. MATHEMATICAL DERIVATIONS FOR THE MONOMER–HOMOTETRAMER–HETEROTETRAMER EQUILIBRIUM MODEL

This section describes the mathematical derivations for the monomer–homotetramer–heterotetramer equilibrium model. This model was used for nonlinear least-squares fitting of the Job plot and for generating simulated Job plots. The mathematical derivations are based on those developed by Collum and co-workers in the supporting information of Liou, L. R; McNeil, A. J.; Ramirez, A.; Toombes, G. E. S.; Gruver, J. M.; Collum, D. B. J. Am. Chem. Soc. 2008, 130, 4859–4868. The following subsections describe (A) the mathematical derivations with general equations; (B) the implementation for homotetramers and heterotetramers; (C) and our implementation for monomers, homotetramers, and heterotetramers.

A. General Equations

In this subsection, I describe the mathematical derivations with general equations. The general equations calculate the concentrations of homooligomers and heterooligomers (that are the same size) as a function of the mole fraction of compounds “A” and “B”:

\[ A_N, A_{N-1}B_1, A_{N-2}B_2, A_{N-3}B_3, \ldots B_N \]

The stoichiometry of the oligomers can be generalized using the term “\(A_nB_{N-n}\)”, where the value of \(N\) reflects oligomer size; the value of \(n\) reflects the number of "A" subunits; and the value of \(N-n\) reflects the number of "B" subunits. For example, \(N = 4\) and \(n = 1\) for an \(A_1B_3\) heterotetramer.
Three main factors influence the relative concentration of oligomers $A_nB_{N-n}$ at equilibrium: multiplicity, free energy, and chemical potential. The equations developed by Collum and co-workers combine these factors for calculating the concentrations of homooligomers and heterooligomers.

1. **Multiplicity ($M_n$)**: The number of ways the “A” and “B” subunits can be arranged within an oligomer $A_nB_{N-n}$. Each unique arrangement is called a permutation ($\rho$). Oligomers that have multiple permutations are present in larger concentrations than oligomers that have only one. The multiplicity or the number of permutations of an oligomer $A_nB_{N-n}$ can be determined with Pascal's triangle or by using binomial theorem, which is shown here:

$$M_n = \frac{N}{(N-n)! \times n!}$$

2. **Free Energy ($g_{\rho}$)**: The relative stability of an oligomer permutation $\rho$. Permutations with the same stoichiometry often have the same relative stability. [In the $A_2B_2$ heterotetramer of peptides 1a and 1b, the $A\cdot A/B\cdot B$ and $A\cdot B/A\cdot B$ topological isomers do not have the same relative stability.] The variable $\phi_{N,n}$ relates the free energy of each permutation to the relative stability.

$$-g_{\rho} = kT \ln (\phi_{N,n})$$

3. **Chemical Potential ($\mu_A$ and $\mu_B$)**: The potential energy associated with the moles of compound “A” and the moles of compound “B” in a mixture. The mole fraction of the compounds reflects the relative chemical potential. The relative chemical potential of $\mu_A$ and $\mu_B$ shifts as the mole fraction of A and B is varied in a Job’s method of continuous variation experiment. For a mixture of tetracaters, when the mole fraction of A is greater than the mole fraction of B, the concentration the $A_3B_1$ heterotetramer is greater than the concentration of the $A_1B_3$ heterotetramer.
To calculate the concentration of a permutation, the free energy and the chemical potential terms are combined to give the following equation:

\[ [\rho] = C \times \exp \left( \frac{-g_\rho + n_\rho \mu_A + (N - n_\rho) \mu_B}{kT} \right) \] (1)

The free energy \( g_\rho \) is the measure of the relative stability of the corresponding permutation \( \rho \); the value of \( n_\rho \) is the number of the “A” subunits within the permutation \( \rho \); the value of \( \mu_A \) is the chemical potential of compound A; the value of \( \mu_B \) is the chemical potential of compound B. The constant \( C \) relates oligomerization propensity to the total concentration.

The concentration of an oligomer \( A_nB_{N-n} \) is the sum of the concentrations of permutations that have the same stoichiometry \( \rho ; n_\rho = n \). For calculating the concentration of an oligomer \( A_nB_{N-n} \), the multiplicity term is combined with the free energy term and chemical potential term to give the following equation:

\[ [A_nB_{N-n}] = \sum_{\rho ; n_\rho = n} [\rho] = C \times \exp \left( \frac{n_\rho \mu_A + (N - n_\rho) \mu_B}{kT} \right) \times \sum_{\rho , n_\rho = n} \exp \left( \frac{-g_\rho}{kT} \right) \] (2)

\[ = C \times \exp \left( \frac{n_\rho \mu_A + (N - n_\rho) \mu_B}{kT} \right) \times M_n \times \langle \exp \left( \frac{-g_\rho}{kT} \right) \rangle_{\rho , n_\rho = n} \] (3)

In this equation, the concentrations of permutations \( \rho \) that have the same stoichiometry \( \rho ; n_\rho = n \) are multiplied by the multiplicity \( M_n \) to give the oligomer concentration \( [A_nB_{N-n}] \). In a Job’s method of continuous variation experiment, the sum of the concentrations of permutations \( \rho \) that have the same stoichiometry \( \rho ; n_\rho = n \) gives the oligomer concentration \( [A_nB_{N-n}] \).
To simplify equation (3), the variables $a$ and $b$ were used to represent the effective chemical potentials $\mu_A$ and $\mu_B$, and the variable $\phi_{N,n}$ was used to represent the relative stability of an oligomer $A_nB_{N-n}$.

$$a = \exp\left(\frac{\mu_A}{kT}\right) \quad b = \exp\left(\frac{\mu_B}{kT}\right) \quad \phi_{N,n} = \langle \exp\left(\frac{g_n}{kT}\right) \rangle_{P_n^P, \eta^P} = n$$

Incidentally, the values of $a$ and $b$ are related to each other such that

$$a + b = 1 \quad \text{and} \quad \frac{a}{b} = \frac{a}{1-a}$$

Incorporation of these variables into equation (3) gives the following equation:

$$[A_nB_{N-n}] = C \times M_n \times \phi_{N,n} \times a^n \times b^{N-n} \quad (4)$$

Equation (4) is the general equation for calculating oligomer concentration. To calculate the relative concentration of an oligomer, the concentration is divided by the sum of the concentrations of all the oligomers $A_jB_{N-j}$:

$$\frac{[A_nB_{N-n}]}{\sum_{j=0}^{N} [A_jB_{N-j}]} = \frac{C \times M_n \times \phi_{N,n} \times a^n \times b^{N-n}}{\sum_{j=0}^{N} C \times M_j \times \phi_{N,j} \times a^j \times b^{N-j}} \quad (5)$$
B. Equations for Homotetramers and Heterotetramers

In this section, I describe the equations for homotetramers and heterotetramers ($N = 4$). Heterotetramers have multiple permutations $\rho$, which increases the concentrations of the heterotetramers relative to the concentrations of the homotetramers. Table S3.3 summarizes the permutations $\rho$ of the homotetramers and heterotetramers.

### Table S3.3. Permutations $\rho$ of the homotetramers and heterotetramers

<table>
<thead>
<tr>
<th>stoichiometry</th>
<th>multiplicity $M_n$</th>
<th>permutation $\rho$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_4 B_{N-n}$</td>
<td>1</td>
<td>AAAA, AAAB, AABA, ABAA, AAAB</td>
</tr>
<tr>
<td>$A_3B_1$</td>
<td>4</td>
<td>AABB, ABAB, BABA, BBAA, ABBA</td>
</tr>
<tr>
<td>$A_2B_2$</td>
<td>6</td>
<td>ABBB, BABB, BBAB, BBBA</td>
</tr>
<tr>
<td>$A_1B_3$</td>
<td>4</td>
<td>BB</td>
</tr>
<tr>
<td>$B_4$</td>
<td>1</td>
<td>BBB</td>
</tr>
</tbody>
</table>

The parameters $\phi_{N,n}$ are ascribed to each of the homotetramers and heterotetramers, where the $N$ and $n$ are integers in which the value of $N$ describes the oligomer size and the value of $n$ describes the number of "A" subunits. The value of each $\phi_{N,n}$ reflects the relative stability of each homotetramer or heterotetramer. The parameters $\phi_{4,4}$, $\phi_{4,3}$, $\phi_{4,2}$, $\phi_{4,1}$, and $\phi_{4,0}$ describe the relative stabilities of $A_4$, $A_3B_1$, $A_2B_2$, $A_1B_3$, and $B_4$, respectively. The following equations are based on equation (4) and contain these parameters for calculating the concentrations of each homotetramer and heterotetramer:

\[
[A_4] = 1 \times C \times \phi_{4,4} \times a^4 \quad (6)
\]
\[
[A_3B_1] = 4 \times C \times \phi_{4,3} \times a^3 b^1 \quad (7)
\]
\[
[A_2B_2] = 6 \times C \times \phi_{4,2} \times a^2 b^2 \quad (8)
\]
\[
[A_1B_3] = 4 \times C \times \phi_{4,1} \times a^1 b^3 \quad (9)
\]
\[
[B_4] = 1 \times C \times \phi_{4,0} \times b^4 \quad (10)
\]
The following equation calculates the relative integration \((I_{N,n})\) by dividing the integration of one tetramer by the sum of the integrations of all tetramers.

\[
I_{N,n} = \frac{C \times M_n \times \phi_{N,n} \times a^n \times b^{N-n}}{\sum_{j=0}^{N} C \times M_j \times \phi_{N,j} \times a^j \times b^{N-j}}
\] (11)

The following equations calculate the relative integration of each homotetramer and heterotetramer:

\[
I_{4,4} = \frac{\phi_{4,4}a^4}{\phi_{4,4}a^4 + 4\phi_{4,3}a^3b + 6\phi_{4,2}a^2b^2 + 4\phi_{4,1}a^1b^3 + \phi_{4,0}b^4}
\] (12)

\[
I_{4,3} = \frac{4\phi_{4,3}a^3b}{\phi_{4,4}a^4 + 4\phi_{4,3}a^3b + 6\phi_{4,2}a^2b^2 + 4\phi_{4,1}a^1b^3 + \phi_{4,0}b^4}
\] (13)

\[
I_{4,2} = \frac{6\phi_{4,2}a^2b}{\phi_{4,4}a^4 + 4\phi_{4,3}a^3b + 6\phi_{4,2}a^2b^2 + 4\phi_{4,1}a^1b^3 + \phi_{4,0}b^4}
\] (14)

\[
I_{4,1} = \frac{4\phi_{4,1}a^1b}{\phi_{4,4}a^4 + 4\phi_{4,3}a^3b + 6\phi_{4,2}a^2b^2 + 4\phi_{4,1}a^1b^3 + \phi_{4,0}b^4}
\] (15)

\[
I_{4,0} = \frac{\phi_{4,0}b}{\phi_{4,4}a^4 + 4\phi_{4,3}a^3b + 6\phi_{4,2}a^2b^2 + 4\phi_{4,1}a^1b^3 + \phi_{4,0}b^4}
\] (16)
C. Equations for Monomers, Homotetramers, and Heterotetramers

In this section, I describe how I modified the equations to accommodate the equilibrium of the monomers with the homotetramers and heterotetramers. The result is the equation used for the monomer–homotetramer–heterotetramer equilibrium model for nonlinear least-squares fitting of the Job plot.

\[ \text{A, B, A}_4, \text{A}_3\text{B}_1, \text{A}_2\text{B}_2, \text{A}_1\text{B}_3, \text{B}_4 \]

I used the following equations to calculate the concentrations of the monomers as a function of their respective relative stabilities \( \phi_{N,a} \) and the chemical potentials \( a \) and \( b \).

\[
[A] = C \times \phi_{1,1} \times a \\
[B] = C \times \phi_{1,0} \times b
\]

(17)  (18)

I used the mass balance equation to accommodate the total concentration of compounds A and B. The total concentration has little or no effect on the equilibria among homotetramers and heterotetramers. By contrast, the total concentration is critical in the equilibria of the monomers with the homotetramers and heterotetramers. The mass balance equation gives the total concentration of compounds A and B (\([A]_{\text{total}} \) and \([B]_{\text{total}} \)) as a function of the monomers, homotetramers, and heterotetramers.

\[
[A]_{\text{total}} + [B]_{\text{total}} = [A] + [B] + 4([A_4] + [A_3B_1] + [A_2B_2] + [A_1B_3] + [B_4])
\]

(19)

Substitution of equations (6), (7), (8), (9), (10), (17), and (18) into the mass balance equation gives the following equation:

\[
[A]_{\text{total}} + [B]_{\text{total}} = C(\phi_{1,1} a + \phi_{1,0} b) + 4C(\phi_{4,4} a^4 + 4\phi_{4,3} a^3 b^1 + 6\phi_{4,2} a^2 b^2 + 4\phi_{4,1} a^1 b^3 + \phi_{4,0} b^4)
\]

(20)
Equation (20) was simplified using the following identities, which represent \(a\) and \(b\) in terms of \(\alpha\) and \((1 - \alpha)\):

\[
\begin{align*}
  a &= a / (a + b) \\
  x &= a + b \\
  a &= \alpha x \\
  b &= (1 - \alpha)x
\end{align*}
\]

Substitution of \(a = \alpha x\) and \(b = (1 - \alpha)x\) into equation (20) gives the following equation:

\[
[A]_{total} + [B]_{total} = xC(\phi_{1,1} \alpha + \phi_{1,0} (1 - \alpha)) + 4x^4 C(\phi_{4,4} \alpha^4 + 4\phi_{4,3} \alpha^3 (1 - \alpha)^1 + 6\phi_{4,2} \alpha^2 (1 - \alpha)^2 + 4\phi_{4,1} \alpha^1 (1 - \alpha)^3 + \phi_{4,0} (1 - \alpha)^4)
\]  

(21)

Equation (21) was simplified by representing the concentrations of the monomers and tetramers in terms of \(M_{total}\) and \(T_{total}\):

\[
\begin{align*}
  M_{total} &= xC(\phi_{1,1} \alpha + \phi_{1,0} (1 - \alpha)) \\
  T_{total} &= 4x^4 C(\phi_{4,4} \alpha^4 + 4\phi_{4,3} \alpha^3 (1 - \alpha)^1 + 6\phi_{4,2} \alpha^2 (1 - \alpha)^2 + 4\phi_{4,1} \alpha^1 (1 - \alpha)^3 + \phi_{4,0} (1 - \alpha)^4)
\end{align*}
\]

Substitution of \(M_{total}\) and \(T_{total}\) into the mass balance equation gives the equation for a monomer–tetramer (monomer–homotetramer–heterotetramer) equilibrium model:

\[
[A]_{total} + [B]_{total} = x M_{total} + 4x^4 T_{total}
\]  

(22)

Setting the equation equal to zero gives the following fourth-order polynomial:

\[
x M_{total} + 4x^4 T_{total} - ([A]_{total} + [B]_{total}) = 0
\]  

(23)

The fourth-order polynomial was solved for \(x\) using Mathematica 10.3 (Wolfram Research, Champaign, IL), which gave a set of four roots (not shown). Each root was evaluated under typical conditions of monomer and tetramer equilibrium (e.g. \(M_{total} = 1.4, T_{total} = 1.65,\) and \(([A]_{total} + [B]_{total}) = 8\)). The root that gave a non-negative value of \(x\) was used as the monomer–homotetramer–heterotetramer equilibrium model.
3.IV. **NONLINEAR LEAST-SQUARES FITTING OF THE JOB PLOT**

This section describes how I used the monomer–homotetramer–heterotetramer equilibrium model for nonlinear least-squares fitting of the Job Plot. To perform the fit, the model was incorporated into a .m script and executed with a series of scripts in MATLAB 2015b. The scripts are based on those developed by Collum and co-workers in the supporting information of Liou, L. R; McNeil, A. J.; Ramirez, A.; Toombes, G. E. S.; Gruver, J. M.; Collum, D. B. *J. Am. Chem. Soc.* **2008**, *130*, 4859–4868.

The following subsections describe the process of fitting the model to our experimental data (Table S3.2) from the Job’s method of continuous variation experiment. The subsections also contain the code from each script along with annotations that describe how the code is used.

```
The code is indicated by bracketing the code text with bars along the left- and right-hand side, as shown here.
```

Annotations for the code appear in between the bracketed text, as shown here.

**A. End User Instructions**

1. Copy the code from each subsection into its own text file, but do not transfer the annotations. Save each file into the same folder or directory using the following file names:

   - `data_Monomer_Tetramer.m`
   - `try_fit.m`
   - `refine_fit.m`
   - `multimers.m`
   - `populations_tetramer.m`
   - `populations_monomer.m`
   - `error_of_model.m`

2. Open MATLAB and navigate the “Current Folder” of the program to the directory where the .m scripts were saved.

3. Load the data from the `data_Monomer_Tetramer.m` file into MATLAB. The data can be loaded in one of two ways: by opening the script with MATLAB and clicking “Run” in the window or by typing the file name into the MATLAB command line and pushing enter.
4. Run the `try_fit.m` script. This script can be run with or without `Expt_Errors`. To run the script without `Expt_Errors`, type the following `try_fit` function into the command line and push enter:

```matlab
try_fit(Xb, Ctotal, phi_monomer, peak_assignment_monomer, phi_tetramer, peak_assignment_tetramer, Expt_Populations)
```
To run the `try_fit.m` script with `Expt_Errors`, type the following `try_fit` function into the command line and push enter:

```matlab
try_fit(Xb, Ctotal, phi_monomer, peak_assignment_monomer, phi_tetramer, peak_assignment_tetramer, Expt_Populations, Expt_Errors)
```

5. The data points in the figure should resemble the data shown in Figure 8. The curves that overlay the data should resemble the curves from the simulated Job plot in Figure 9e.

6. To run the `refine_fit.m` script without `Expt_Errors`, type the following `refine_fit` function into the command line and push enter:

```matlab
refine_fit(Xb,Ctotal,phi_monomer, peak_assignment_monomer, phi_tetramer, peak_assignment_tetramer, Expt_Populations, phi_constant)
```
To run the `refine_fit.m` script with `Expt_Errors`, type the following `refine_fit` function into the command line and push enter:

```matlab
refine_fit(Xb,Ctotal,phi_monomer, peak_assignment_monomer, phi_tetramer, peak_assignment_tetramer, Expt_Populations, phi_constant, Expt_Errors)
```
After the fit, the final phi values shown in the MATLAB terminal are the optimized phi values. These values should be comparable the phi values listed in Figure 8.

7. Make a copy of the file `data_Monomer_Tetramer.m` to a new file called `data_Monomer_Tetramer_new.m`. Replace the phi values with the optimized values from the `refine_fit.m` script.

8. Load the new data file `data_Monomer_Tetramer_new.m` into MATLAB. Type the `try_fit` function into the command line and push enter to observe the optimized fit.
B. Definitions
1. \( X_b(j) \) is the mole fraction \( \chi_B \).
2. \( C_{\text{total}} \) is the input for the total concentration of each mixture.
3. \( \text{Expt\_Populations} \) is the experimental data input for the relative integrations of the monomers, homotetramers, and heterotetramers (Table S3.2). These data are referred to as the “experimental populations”.
4. \( \text{Expt\_Errors} \) is the input for the error of the measurements.
5. \( \text{peak\_assignment\_monomer} \) and \( \text{peak\_assignment\_tetramer} \) are column identifiers assigned to each monomer and tetramer population.
6. \( \phi_{\text{monomer}} \) and \( \phi_{\text{tetramer}} \) are measures of the relative stabilities of the monomers and tetramers. These values are assigned to each monomer, homotetramer, and heterotetramer population and are used by the monomer–homotetramer–heterotetramer equilibrium model for calculating the relative concentrations of each population.
7. \( \phi_{\text{constant}} \) is the input that dictates whether a \( \phi_{\text{monomer}} \) or \( \phi_{\text{tetramer}} \) value remains fixed or is allowed to vary during nonlinear least-squares fitting. A value of 1 allows the corresponding \( \phi \) to vary; a value of 0 keeps the corresponding \( \phi \) fixed.
8. \( \text{Expt\_weights} \) is the input for the error of each data point and is used for weighting the error of each data point. The data points are weighted equally if nothing is entered.
9. \( \text{conc\_monomer} \) and \( \text{conc\_tetramer} \) are used for calculating and storing the concentrations for each monomer, homotetramer, and heterotetramer population. [Note that even though the term concentration is used, the scripts are actually calculating the integrations of the monomer and tetramer populations.]
10. \( \text{pop\_monomer} \) and \( \text{pop\_tetramer} \) are used to temporarily store calculated values for the concentrations (relative integrations) of each monomer or tetramer population. These data are referred to as the calculated (predicted) populations.
11. \( \text{Model\_Populations} \) is the final output for the concentrations (relative integrations) of the monomers, homotetramers, and heterotetramers.
12. \( \text{mean\_error} \) weighted standard deviation of the residuals over the entire fit.
13. \( \text{pop\_error}(1,j) \) is the mean error of experimental populations – calculated populations. The value could be negative, zero, or positive.
14. \( \text{pop\_error}(2,j) \) is the root mean square error of experimental populations – calculated populations. The value is always positive.
15. \( \phi_{\text{dimer\_new}} \) and \( \phi_{\text{tetramer\_new}} \) are the new values of each \( \phi \) after the fit.
16. \( \text{error} \) is the root mean square error of the new calculated populations.
C. Monomers, Homotetramers, and Tetramers: Data

This script stores the experimental populations and the initial values for performing the fit.

This code clears all stored information in the command line and closes all figures.

```matlab
clear variables;
close all;
clc;
```

This code is the input for the total concentration of the mixtures, which is designated Ctotal. The number of values in Ctotal equal the number of samples studied. In this case, nine samples were studied.

```matlab
Ctotal = [0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008 0.008];
```

This code is the input the mole fraction $\chi_B$, which is designated Xb. The values entered in Xb equal the mole fraction of each mixture studied. The values are listed from lowest to highest.

```matlab
Xb = [0.00 0.125 0.25 0.375 0.50 0.625 0.75 0.875 1.00];
```

This code is the input for the experimental populations: the relative integrations from the Job’s method of continuous variation experiment (Table S3.2). Each column lists the relative integrations of the monomer and tetramer populations in the following order: A, B, A₄, A₃B₁, A₂B₂, A₁B₃, B₄. The columns are separated by a space. Each row lists the relative integrations of the mole fractions Xb listed in the following order: 0.00, 0.125, 0.25, 0.375, 0.50, 0.625, 0.75, 0.875, and 1.00. The rows are separated by a semicolon.

```matlab
Expt_Populations = [
    0.0664   0.0000  0.9336  0.0000  0.0000  0.0000  0.0000;
    0.0449   0.0608  0.6883  0.1183  0.0873  0.0003  0.0000;
    0.0332   0.1089  0.4359  0.1459  0.2654  0.0102  0.0005;
    0.0281   0.1445  0.2986  0.1391  0.3639  0.0216  0.0042;
    0.0177   0.1987  0.1350  0.1057  0.4640  0.0581  0.0209;
    0.0115   0.2699  0.0608  0.0695  0.4748  0.0751  0.0384;
    0.0082   0.3095  0.0096  0.0260  0.3516  0.1486  0.1466;
    0.0044   0.3820  0.0009  0.0060  0.1595  0.1731  0.2741;
    0.0000   0.4796  0.0000  0.0000  0.0000  0.0000  0.5204];
```
The Expt_Errors input is optional for the fit. These values should be listed for the monomer and
tetramer populations in the following order: A, B, A_4, A_3B_1, A_2B_2, A_1B_3, B_4, which is the same
order used for the populations in the Expt_Populations input.

\%
Expt_Errors = [ ];

The peak_assignment_monomer and peak_assignment_tetramer are inputs that designate the
column for each monomer or tetramer. For a given monomer or tetramer, the assignment value
specifies which column the data should be read from or stored in. The
peak_assignment_monomer values are listed in the following order: A, B; the
peak_assignment_tetramer values are listed in the following order: A_4, A_3B_1, A_2B_2, A_1B_3, B_4.

\[
\text{peak_assignment_monomer} = [1 \ 5 ]; \\
\text{peak_assignment_tetramer} = [ 1 \ 2 \ 3 \ 4 \ 5 ];
\]

The initial phi values are all set to one.

The phi_monomer values are listed in the following order: A, B; the phi_tetramer values are
listed in the following order: A_4, A_3B_1, A_2B_2, A_1B_3, B_4.

\[
\text{phi_monomer} = [1 \ 1 ]; \\
\text{phi_tetramer} = [1 \ 1 \ 1 \ 1 ];
\]

The phi_constants that are set to 1 allow the phi value to be refined with the refine_fit.m script;
the phi_constants that are set to 0 keep the value fixed during the refine_fit.m script.

The phi_constant values are listed in the following order: A, B, A_4, A_3B_1, A_2B_2, A_1B_3, B_4. The
phi_constant for the A_4 homotetramer was fixed so that the refine_fit.m script gives a unique
solution.

\[
\text{phi_constant} = [1 \ 1 \ 0 \ 1 \ 1 \ 1 ];
\]
**D. Monomers, Homotetramers, and Tetramers: Try Fit**

This script plots the experimental populations, and also plots the populations calculated from the phi values. The two plots are overlayed in a new window. The experimental populations are plotted versus the mole fraction \(X_b\) as open circles; the calculated populations are plotted versus the mole fraction \(X_b\) as smooth lines. The script also determines the error between the experimental populations and the calculated populations then prints these values in the MATLAB terminal.

```matlab
function try_fit(Xb, Ctotal,...
    phi_monomer, peak_assignment_monomer,...
    phi_tetramer, peak_assignment_tetramer,...
    Expt_Populations, Expt_Errors)

This code determines whether Expt_Errors are entered.

```matlab
    if(nargin<8)
        Expt_weights=ones(size(Expt_Populations));
    else
        Expt_weights=1./((Expt_Errors+mean(mean(Expt_Errors))));
    end
```

This code plots the experimental populations of the monomers and tetramers from the Expt_Populations input.

```matlab
    hold on ; cscheme= 'kybmgerkymbmgc'; axis([0 1 0 1]); xlabel('X_B');
    ylabel('Relative Integration');
    for j=1:size(Expt_Populations,2)
        if (nargin<8)
            plot(Xb, Expt_Populations(:,j),sprintf('%so',cscheme(j)));
        else
            errorbar(Xb, Expt_Populations(:,j), Expt_Errors(:,j),sprintf('%so',cscheme(j)));
        end
    end
```

This code calculates the monomer and tetramer populations using the initial values of the phi's entered.
XBc = (0:0.01:1);
Ctotalac = Ctotal(1)*ones(size(XBc));
[conc_monomers, conc_tetramers] = multimers(XBc, Ctotalac,...
    phi_monomer, phi_tetramer);

This code stores the calculated monomer and tetramer populations and stores them in a matrix.

    pop_tetramer = populations_tetramer(conc_monomers,...
        peak_assignment_monomer, conc_tetramers, peak_assignment_tetramer);
    pop_monomer = populations_monomer(conc_monomers,...
        peak_assignment_monomer, conc_tetramers, peak_assignment_tetramer);
    pop_tetramer_corrected = pop_tetramer-pop_monomer;
    pop_combined = horzcat(pop_monomer(:,1),...
        pop_monomer(:,5),pop_tetramer_corrected);

This code plots the calculated populations.

    for j=1:size(pop_combined,2)
        plot(XBc,pop_combined(:,j),sprintf('%c',cscheme(j)) );
    end

This code compares the experimental and the calculated populations, then calculates and displays
the error.

    [mean_error, pop_error] = error_of_model(Xb, Ctotal,...
        phi_monomer, peak_assignment_monomer,...
        phi_tetramer, peak_assignment_tetramer,...
        Expt_Populations, Expt_weights);

    N = length(horzcat(phi_monomer, phi_tetramer)) - 1;
    fprintf(1,'\nThe Mean mismatch is %f percent.\n', mean_error*100);

    for j=1:size(pop_error, 2)
        fprintf(1,'Predicted value of Population %d exceeds measurement by %f percent
and mean square error of %f percent.\n',j, pop_error(1,j)*100,pop_error(2,j)*100);
    end
E. Monomers, Homotetramers, and Tetramers: Refine Fit

This script performs the nonlinear least-squares fitting. The script optimizes the phi values to match the calculated populations to the experimental populations. The script reports the new phi values and the root mean square difference between the calculated and the experimental populations.

```matlab
function [phi_dimer_new, phi_tetramer_new, error] = refine_fit(Xb,Ctotal,...
    phi_monomer, peak_assignment_monomer,...
    phi_tetramer, peak_assignment_tetramer,...
    Expt_Populations, phi_constant, Expt_Errors)

This code determines whether Expt_errors are entered.

    if (nargin<9)
        Expt_weights = ones(size(Expt_Populations));
    else
        Expt_weights = 1./( Expt_Errors + mean(mean(Expt_Errors)));
    end

This code merges the monomer Expt_Populations and the tetramer Expt_Populations into a single input.

    phimerge = [phi_monomer, phi_tetramer];
    idx_monomer = [1 2]; idx_tetramer = [3 4 5 6 7];
    param = [1:length(phimerge)];

This code sets the initial step size used to optimize the phi values; the initial step size is 10%.

    step_size = 0.1*phi_constant.*phimerge(param);
    N_no_progress = 0;
    N_max_trials = 30;
```
This code compares the calculated and experimental populations of the monomers and tetramers, then calculates and displays the error of the model.

```matlab
[error_best, temp] = error_of_model(Xb, Ctotal,...
    phimerge(idx_monomer), peak_assignment_monomer,...
    phimerge(idx_tetramer), peak_assignment_tetramer,...
    Expt_Populations, Expt_weights);
    
    fprintf(1, 'n Initial Error of Fit = %f percent\n', error_best * 100);
```

This code is a "for while" loop that reduces the error of the model by optimizing the phi values.

```matlab
while (N_no_progress < N_max_trials)
    flag = 0;
    for k=1:length(param)

This code adjusts the value of phi to the "right" and to the "left".

```matlab
    phi_testr = phimerge;
    phi_testr(param(k)) = abs(phimerge(param(k)) + step_size(k));
    [error_testr, temp] = error_of_model(Xb, Ctotal,...
        phi_testr(idx_monomer), peak_assignment_monomer,...
        phi_testr(idx_tetramer), peak_assignment_tetramer,...
        Expt_Populations, Expt_weights);

    phi_testl = phimerge;
    phi_testl(param(k)) = abs(phimerge(param(k)) - step_size(k));
    [error_testl, temp] = error_of_model(Xb, Ctotal,...
        phi_testl(idx_monomer), peak_assignment_monomer,...
        phi_testl(idx_tetramer), peak_assignment_tetramer,...
        Expt_Populations, Expt_weights);
```
This code determines which adjustment of phi decreases the error of the model. If either the right or the left value decreases the error, then that value is stored and the loop repeats again. If neither the right or the left value decreases the error, then the step is flagged and the size of the step is reduced.

```matlab
if (error_testr < error_best)
    error_best = error_testr;
    phimerge = phi_testr;
    step_size(k) = step_size(k) * 1.5;
    N_no_progress = 0;
elseif (error_testl < error_best)
    error_best = error_testl;
    phimerge = phi_testl;
    step_size(k) = step_size(k) * 1.5;
    N_no_progress = 0;
else
    flag = flag + 1;
end
end
if (flag >= length(param))
    step_size = step_size * (0.75 + 0.25 * rand);
    N_no_progress = N_no_progress + 1;
end
This code displays the new fit after each phi has been adjusted

```matlab
fprintf(1,'\n
 Error - %f , Last Good Step - %d , Mean Step Size - %f... ,error_best, N_no_progress, 100*mean(step_size) );
fprintf(\n Phi Monomer - '); fprintf(1,'%f ', phimerge(idx_monomer));
fprintf(1,'\n Phi Tetramer - '); fprintf(1,'%f ',phimerge(idx_tetramer));
end

error = error_best;
phi_monomer_new = phimerge(idx_monomer);
phi_tetramer_new = phimerge(idx_tetramer);
```
F. Monomers, Homotetramers, and Tetramers: Multimers

For each mole fraction $X_b$, this script calculates the concentrations (relative integrations) of the monomer and tetramer populations using the inputs: $X_b$, $C_{total}$, $\phi_{monomer}$, and $\phi_{tetramer}$.

```matlab
function [conc_monomer, conc_tetramer] = multimers(Xb, Ctotal,...
    phi_monomer, phi_tetramer)
for j=1:length(Xb)
    [conc_monomer(j,:), conc_tetramer(j,:)] = bisect(Xb(j), Ctotal(j),... 
        phi_monomer, phi_tetramer);
end
```

This code is the bisection function, which optimizes the "relative chemical potential" until the value reflects the mole fraction of the experimental mole fraction.

```matlab
function [conc_monomer, conc_tetramer] = bisect(Xb, Ctotal,...
    phi_monomer, phi_tetramer)

tolerance = 1e-6;
bmax = 1; bmin = 0;
[Xmin, conc_monomer, conc_tetramer] = Cparametric(bmin,...
    phi_monomer, phi_tetramer, Ctotal);
[Xmax, conc_monomer, conc_tetramer] = Cparametric(bmax,...
    phi_monomer, phi_tetramer, Ctotal);

while ((Xmax - Xb) > tolerance)
    btest = (bmin + bmax) / 2;
    [Xtest, conc_monomer, conc_tetramer] = Cparametric(btest, phi_monomer,...
        phi_tetramer, Ctotal);

    if (Xtest > Xb)
        bmax = btest; Xmax = Xtest;
    else
        bmin = btest; Xmin = Xtest;
    end
end
```
This code is the mathematical model for the monomer and tetramer equilibrium.

```matlab
function [Xb, conc_monomer, conc_tetramer] = Cparametric(b, phi_monomer, phi_tetramer, Ctotal )
    a = 1 - b;
    Tscale = 1e9;
    Mtotal = (phi_monomer(1)*a + phi_monomer(2)*b);
    Ttotal = Tscale * (phi_tetramer(1) * a^4 + 4 * phi_tetramer(2) * a^3 * b +...
        6 * phi_tetramer(3) * a * a * b * b + 4 * phi_tetramer(4) * a * b^3 +...
        phi_tetramer(5)*b^4);
    Chi = ((1/2)*sqrt(-((9*Ttotal*Mtotal^2+sqrt(3)*sqrt(27*Ttotal^2*Mtotal^4+1024*Ttotal^3*Ctotal^3))^(1/3)/(2*6^(2/3)*Ttotal)+Mtotal/(sqrt(2)*...
        Ttotal*sqrt((9*Ttotal*Mtotal^2+sqrt(3)*sqrt(27*Ttotal^2*Mtotal^4+1024*Ttotal^3*Ctotal^3))^(1/3)/(6^(2/3)*Ttotal)+4*2^(2/3)*Ctotal)/(3^(1/3)*...
        (9*Ttotal*Mtotal^2+sqrt(3)*sqrt(27*Ttotal^2*Mtotal^4+1024*Ttotal^3*Ctotal^3))^(1/3))))(2*2^(2/3)*Ctotal)/(3^(1/3)*(9*Ttotal*Mtotal^2+...
        sqrt(3)*sqrt(27*Ttotal^2*Mtotal^4+1024*Ttotal^3*Ctotal^3))^(1/3)))+...
    sqrt((9*Ttotal*Mtotal^2+sqrt(3)*sqrt(27*Ttotal^2*Mtotal^4+1024*Ttotal^3*Ctotal^3))^(1/3)/(3^(1/3)*(9*Ttotal*Mtotal^2+...
        sqrt(3)*sqrt(27*Ttotal^2*Mtotal^4+1024*Ttotal^3*Ctotal^3))^(1/3)))+...
    sqrt((9*Ttotal*Mtotal^2+sqrt(3)*sqrt(27*Ttotal^2*Mtotal^4+1024*Ttotal^3*Ctotal^3))^(1/3))/...
    (2*sqrt(2)))
    conc_monomer = Chi/Ctotal*[phi_monomer(1)*a, phi_monomer(2)*b];
    conc_tetramer = Tscale * 4 * Chi^4/Ctotal*[phi_tetramer(1)*a*a*a*a,...
        4*phi_tetramer(2)*a*a*a*b, 6*phi_tetramer(3)*a*a*b*b,...
        4*phi_tetramer(4)*a*b*b*b, phi_tetramer(5)*b*b*b*b];
    Xb = sum(conc_monomer.*[0 1])+sum(conc_tetramer.*[0 0.25 0.5 0.75 1]);
```
G. Monomers, Homotetramers, and Tetramers: Tetramer Populations

For all mole fractions Xb in the calculation, the populations_tetramer script stores all of the calculated populations for the tetramers.

```matlab
function result = populations_tetramer(conc_monomer, peak_assignment_monomer,...
    conc_tetramer, peak_assignment_tetramer)

    result = zeros(size(conc_monomer,1), max(max(peak_assignment_monomer),...
    max(peak_assignment_tetramer)));
    N = size(conc_monomer,2);
    for j=1:N
        idx = peak_assignment_monomer(j);
        result(:,idx) = result(:,idx) + conc_monomer(:,j);
    end

    N = size(conc_tetramer,2);
    for j=1:N
        idx = peak_assignment_tetramer(j);
        result(:,idx) = result(:,idx) + conc_tetramer(:,j);
    end
```

H. Monomers, Homotetramers, and Tetramers: Monomer Populations

For all mole fractions Xb in the calculation, the Populations_monomer script stores all of the calculated populations for the tetramers.

```matlab
function pop_monomer = populations_monomer(conc_monomer,...
    peak_assignment_monomer, conc_tetramer, peak_assignment_tetramer)

    result = zeros(size(conc_monomer,1), max(max(peak_assignment_monomer),...
    max(peak_assignment_tetramer)));
    N = size(conc_monomer,2);
    for j=1:N
        idx = peak_assignment_monomer(j);
        result(:,idx) = result(:,idx) + conc_monomer(:,j);
    end

    pop_monomer = result;
    N = size(conc_tetramer,2);
    for j=1:N
        idx = peak_assignment_tetramer(j);
        result(:,idx) = result(:,idx) + conc_tetramer(:,j);
    end
```
I. Monomers, Homotetramers, and Tetramers: Error of Model

This script is called within the try_fit.m and in the refine_fit.m scripts. The script reports the weighted mean error and population error of the fit.

```matlab
function [mean_error, pop_error] = error_of_model(Xb, Ctotal,...
    phi_monomer, peak_assignment_monomer,...
    phi_tetramer, peak_assignment_tetramer,...
    Expt_Populations, Expt_Errors)

    if (nargin<8)
        Expt_weights=ones(size(Expt_Populations));
    else
        Expt_weights = 1./(Expt_Errors + mean(mean(Expt_Errors)));
    end

    [conc_monomers, conc_tetramers] = multimers(Xb,...
        Ctotal, phi_monomer, phi_tetramer);

    pop_tetramer = populations_tetramer(conc_monomers,...
        peak_assignment_monomer, conc_tetramers, peak_assignment_tetramer);

    pop_monomer = populations_monomer(conc_monomers,...
        peak_assignment_monomer, conc_tetramers, peak_assignment_tetramer);

    pop_tetramer_corrected = pop_tetramer-pop_monomer;

    Model_Populations = horzcat(pop_monomer(:,1),...
        pop_monomer(:,5),pop_tetramer_corrected);

    sizeof_Model = size(Model_Populations);
    sizeof_Expt = size(Expt_Populations);

    Model_Populations_Combined = horzcat(conc_monomers, conc_tetramers);
    sizeof_Model_Combined = size(Model_Populations_Combined);

    diff = Model_Populations_Combined-Expt_Populations;
    mean_error = sqrt(sum(sum(diff.*diff.*Expt_weights)) / sum(sum(Expt_weights)));
    pop_error = sum(diff.*Expt_weights,1) ./ sum(Expt_weights,1);
    pop_error(2,:) = sqrt(sum(diff.*diff.*Expt_weights,1) ./ sum(Expt_weights,1));
```

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3.V. REFERENCES


3.V. CHARACTERIZATION DATA
$^1$H NMR DOSY of a 1:1 mixture of peptides 1a and 1b
8.0 mM total concentration in D$_2$O at 500 MHz and 298 K

Calculations for the 1:1 mixture of peptides 1a and 1b at 8.0 mM total concentration

$D_{HOD} = 19.0 \times 10^{-10} \text{ m}^2/\text{s}$

$log (D_{HOD}) = -8.721$

$D_{\text{heterotetramers}}: log(D) = -9.943; D = 10^{-9.943} = 11.4 \pm 1.1 \times 10^{-11} \text{ m}^2/\text{s}$

$^{1}H,^{15}N$ HSQC of peptides $[^{15}N]1a$ and $[^{15}N]1b$ at 600 MHz and 293 K

$\chi_B = 0.00; \ 8.0 \ mM$ total concentration in 9:1 $H_2O/D_2O$

$^a \chi_B$ designates the mole fraction of peptide $[^{15}N]1b$. 
$^{1}H,^{15}N$ HSQC of peptides $[^{15}N]1a$ and $[^{15}N]1b$ at 600 MHz and 293 K
$\chi_{B}^{a} = 0.125$; 8.0 mM total concentration in 9:1 H$_2$O/D$_2$O

$^{a}$ $\chi_{B}$ designates the mole fraction of peptide $[^{15}N]1b$.
The asterisks (*) indicate crosspeaks associated with minor unidentified species.
$^1$H, $^{15}$N HSQC of peptides $[^{15}$N]1a and $[^{15}$N]1b at 600 MHz and 293 K
$\chi_B = 0.25$; 8.0 mM total concentration in 9:1 H$_2$O/D$_2$O

$^a$ $\chi_B$ designates the mole fraction of peptide $[^{15}$N]1b.
$^1$H,$^{15}$N HSQC of peptides $[^{15}$N]$^1$a and $[^{15}$N]$^1$b at 600 MHz and 293 K
$\chi_B^a = 0.375$; 8.0 mM total concentration in 9:1 H$_2$O/D$_2$O

$^a \chi_B$ designates the mole fraction of peptide $[^{15}$N]$^1$b.
The asterisks (*) indicate crosspeaks associated with minor unidentified species.
$^1\text{H},^{15}\text{N}$ HSQC of peptides $[^{15}\text{N}]1\text{a}$ and $[^{15}\text{N}]1\text{b}$ at 600 MHz and 293 K

$\chi_{B}^a = 0.50; 8.0$ mM total concentration in 9:1 H$_2$O/D$_2$O

$^a$ $\chi_B$ designates the mole fraction of peptide $[^{15}\text{N}]1\text{b}$.
The asterisks (*) indicate crosspeaks associated with minor unidentified species.
$^{1}H,^{15}N$ HSQC of peptides $[^{15}N]1a$ and $[^{15}N]1b$ at 600 MHz and 293 K
$\chi_B^a = 0.625$; 8.0 mM total concentration in 9:1 $H_2O/D_2O$

$^a\chi_B$ designates the mole fraction of peptide $[^{15}N]1b$. 
$^{1}H,^{15}N$ HSQC of peptides $[^{15}N]1a$ and $[^{15}N]1b$ at 600 MHz and 293 K

$\chi_B^a = 0.75$; 8.0 mM total concentration in 9:1 H$_2$O/D$_2$O

$^a \chi_B$ designates the mole fraction of peptide $[^{15}N]1b$. 
$^1$H,$^{15}$N HSQC of peptides $[^{15}$N]$^1$a and $[^{15}$N]$^1$b at 600 MHz and 293 K

$\chi_B = 0.875$; 8.0 mM total concentration in 9:1 H$_2$O/D$_2$O

\( ^a \) $\chi_B$ designates the mole fraction of peptide $[^{15}$N]$^1$b.
$^1$H, $^{15}$N HSQC of peptides $[^{15}$N]$^1$a and $[^{15}$N]$^1$b at 600 MHz and 293 K

$\chi_B^a = 1.00$; 8.0 mM total concentration in 9:1 H$_2$O/D$_2$O

$^a$ $\chi_B$ designates the mole fraction of peptide $[^{15}$N]$^1$b.
$^{1}H,^{15}N$ HSQC of peptides $^{[15N]}1a$ and $^{[15N]}1b$ in 9:1 H$_2$O/D$_2$O at 600 MHz and 293 K
Stack of $^{15}N$ spectra from the $i$ projections of the $^{1}H,^{15}N$ HSQC spectra

$\chi_B^{\text{d}}$

0.00

0.125

0.25

0.375

0.50

0.625

0.75

0.875

1.00

$^a\chi_B$ designates the mole fraction of peptide $^{[15N]}1b$. 

ppm 130 125 120 115 110
CHAPTER 4

Efforts to Correlate the Structure, Solution-Phase Behavior, and Toxicity of Peptides Derived from Aβ_{17–36}
Aggregation of the β-amyloid peptide (Aβ) to form oligomers and fibrils is central to the pathology of Alzheimer’s disease. The fibrils are insoluble deposits of Aβ that consist of layers of β-sheets, with extensive intermolecular hydrogen bonds and hydrophobic contacts. The oligomers are also rich in β-sheet structure, but they consist of dimers and trimers that can further assemble to give higher-order oligomers. While the fibrils represent the thermodynamic endpoint of Aβ aggregation, the oligomers have a fundamental role in causing synaptic dysfunction and neurodegeneration.

The importance of Aβ oligomers in Alzheimer’s disease pathology underscores the significance for obtaining their high-resolution structures. Aβ oligomers form a polymorphic and heterogeneous mixture of aggregates that are difficult to characterize and study. Many of the oligomers show toxicity toward neuronal cells, but efforts to isolate and characterize these oligomers have provided limited structural information. To date, little is known about the structures of Aβ oligomers at high resolution. As a result, the mechanisms are not fully understood by which the oligomers form and contribute to Alzheimer’s disease pathology.

Our laboratory has been using macrocyclic β-sheet peptides to shed light on the oligomers formed by Aβ and other peptides and proteins. In 2014, our laboratory introduced macrocyclic β-sheet peptides derived from a β-hairpin of Aβ17–36. We incorporated the fragments Aβ17–23 (LVFFAED) and Aβ30–36 (AIIGLMV) into the upper and lower strands of a macrocyclic β-sheet peptide, and connected the two strands with δ-linked ornithine (δOrn) turn units. These turn units allow β-sheet folding between the two strands (Figure 4.1). The peptides also contain a single N-methyl amino acid, which prevents uncontrolled aggregation. We began studies of peptide 1Met, which embodies these design features, but we found that this peptide
exhibited limited solubility in aqueous solution. Our laboratory then incorporated an ornithine (α-linked) residue to give peptide $1_{\text{Orn}}$, which we envisioned as a homologue of peptide $1_{\text{Met}}$ that would have better solubility.

![Chemical structures of an Aβ17–36 β-hairpin and peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$ (A–C). Met$_{35}$ and Orn$_{35}$ residues illustrating the relationship between peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$ (D).](image)

**Figure 4.1.** Chemical structures of an Aβ17–36 β-hairpin and peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$ (A–C). Met$_{35}$ and Orn$_{35}$ residues illustrating the relationship between peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$ (D).

Previous X-ray crystallography studies of peptide $1_{\text{Orn}}$ showed that this peptide can form trimers, hexamers, and dodecamers. These oligomers inspired our laboratory to design related analogues derived from Aβ, including covalently-stabilized trimers, larger β-hairpins, and alternative pairings of Aβ residues. X-ray crystallography studies of these peptides show that they can form various sizes and morphologies of dimers, trimers, hexamers, dodecamers, and other higher-order oligomers.
Solution-phase biophysical and biological studies of macrocyclic β-sheet peptides have begun to reveal relationships between oligomerization and toxicity. In a related study of a macrocyclic β-sheet peptide derived from Aβ16–36, our laboratory used X-ray crystallography and SDS-PAGE to show that this peptide can form stable hexamers, and used biological studies showed that these hexamers are toxic to neuronal cells.20 When our laboratory similarly attempted to study the solution-phase behavior and toxicity of peptide 1Orn, we found that peptide 1Orn was largely monomeric. As a result, the biological properties of peptide 1Orn were not explored.

Inspired by the oligomerization and toxicity of the macrocyclic β-sheet peptide derived from Aβ16–36, I decided to revisit the study of the peptides derived from Aβ17–36, peptides 1Orn and 1Met. I begin this study by elucidating the X-ray crystallographic structure of peptide 1Met. After I established that peptide 1Met adopts the same X-ray crystallographic structure as peptide 1Orn, I then attempt to determine whether peptides 1Met and 1Orn form oligomers in solution. I also evaluate the toxicity of these two peptides. Throughout the course of these studies, I compared the oligomers formed by peptides 1Met and 1Orn with those of trimer 2 (Figure 4.2). Trimer 2, which our laboratory previously characterized, is a covalently-stabilized trimer that assembles in solution to form a dimer.17 The monomer and dimer of trimer 2 are comparable in molecular weight to a trimer and hexamer formed by peptides 1Met and 1Orn.
I conclude this chapter with $^1$H NMR spectroscopy studies of peptides 1Met, 1Orn, and trimer 2. I found that peptide 1Met was not soluble at an adequate concentration to produce sufficient NMR signals, while peptide 1Orn and trimer 2 were easily soluble for NMR studies. The $^1$H NMR spectra of peptide 1Orn and of trimer 2 showed that oligomers form, but the oligomer spectra were largely indecipherable. Diffusion-ordered spectroscopy (DOSY) revealed that peptide 1Orn and trimer 2 form oligomers consistent with dodecamers and tetramers, respectively. To promote the formation of a well-defined dodecamer, I also designed analogues of peptide 1Orn (peptides 3 and 4) and studied them by $^1$H NMR spectroscopy and DOSY.

RESULTS AND DISCUSSION

X-ray Crystallographic Structure of Peptide 1Met. Although peptide 1Met exhibits limited solubility, I found that after sonication this peptide is soluble in a solution without buffer at about 5 mg/mL. This concentration proved sufficient for growing crystals of peptide 1Met for X-ray
crystallography. Diffraction data of peptide $\text{1Met}$ were collected in house on a Rigaku MicroMax 007HF X-ray diffractometer at 1.54 Å wavelength. The data were collected to 2.03 Å resolution, processed in XDS, and solved by isomorphous replacement using the structure of peptide $\text{1Orn}$ (PDB ID: 4NTR). The structure of peptide $\text{1Met}$ was processed and refined in the R3 space group. The asymmetric unit contains 16 peptides that adopt folded $\beta$-sheets with seven to eight intramolecular hydrogen bonds. The structures of peptides $\text{1Met}$ and $\text{1Orn}$ are nearly identical, with a root-mean squared deviation of $< 1$ Å. Figure 4.3 shows monomer subunits from the structures of peptides $\text{1Met}$ and $\text{1Orn}$.

![Figure 4.3](image)

**Figure 4.3.** Monomer subunits from the X-ray crystallographic structures of peptide $\text{1Met}$ (A) and peptide $\text{1Orn}$ (B, PDB ID: 4NTR).

The X-ray crystallographic structure shows peptide $\text{1Met}$ assembles to form triangular trimers, which further assemble into hexamers and dodecamers. The alternating side chains of peptide $\text{1Met}$ make up two surfaces—called the major and minor surfaces. The major surface consists of the side chains of Leu$_{17}$, Phe$_{19}$, Ala$_{21}$, Asp$_{23}$, Ala$_{30}$, Ile$_{32}$, Leu$_{34}$, and Val$_{36}$; the minor surface consists of the
side chains of Val$_{18}$, Phe$_{20}$, Ile$_{31}$, Gly$_{33}$, and Met$_{35}$. In the trimer assembly, these residues also create major and minor surfaces. Figure 4.4 shows these two surfaces of the trimer.

**Figure 4.4.** Trimer of peptide 1$_{Met}$ showing select residues as spheres. (A) Major surface showing the residues Leu$_{17}$, Phe$_{19}$, Asp$_{23}$, Ile$_{32}$, Leu$_{34}$, and Val$_{36}$. (B) Minor surface showing the residues Val$_{18}$, Phe$_{20}$, Glu$_{22}$, Ile$_{31}$, and Met$_{35}$. 
The trimer is held together with intermolecular hydrogen-bonds and hydrophobic contacts at the interface of each peptide (Figure 4.5A). This interface shows a pair of hydrogen bonds between Val$_{18}$ and Glu$_{22}$. This interface also shows a group of hydrophobic contacts. On the major surface of the trimer, these hydrophobic contacts come from the side chains of Leu$_{17}$, Phe$_{19}$, and Val$_{36}$ from one peptide and the side chains of Ala$_{21}$, Ile$_{32}$, and Leu$_{34}$ from the adjacent peptide. On the minor surface, these hydrophobic contacts come from the side chains of Val$_{18}$ and Phe$_{20}$ from one peptide and the side chain of Ile$_{31}$ from the adjacent peptide. Three ordered water molecules are located in the center of the trimer, forming hydrogen bonds to the backbone NH and CO groups of Phe$_{20}$. The hexamer is composed of two trimers of peptide 1Met that laminate together on their minor surfaces to form a sandwich-like hexamer (Figure 4.5B). The two trimers are staggered by about 60°. The side chains of Val$_{18}$, Phe$_{20}$, and Ile$_{32}$ point toward the center of the hexamer, forming a hydrophobic interface. The dodecamer is composed of four trimers that come together at the vertices of a tetrahedron (Figure 4.5C). The dodecamer is held together through hydrophobic contacts and a salt bridge at the interface of the four trimers. The salt bridge forms between an Asp$_{23}$ and an α-amine of a δ-linked ornithine. The hydrophobic contacts form between the side chains of Leu$_{17}$, Phe$_{19}$, Val$_{36}$, Leu$_{34}$. 
Figure 4.5. X-ray crystallographic structure of peptide 1_{Met}. Triangular trimer with the three ordered water molecules shown in the center (A). The hexamer (B) and dodecamer (C) that are observed in the crystal lattice.
Solution-Phase Biophysical Studies of peptides 1\text{Met} and 1\text{Orn}. Solution-phase biophysical studies of peptides 1\text{Met} and 1\text{Orn} are important for evaluating whether these peptides fold and oligomerize in aqueous solution. The following subsections evaluate and compare the folding and oligomerization of peptides 1\text{Met} and 1\text{Orn} by circular dichroism spectroscopy and SDS-PAGE.

Circular Dichroism Spectroscopy. Circular dichroism spectroscopy provides a simple way to evaluate folding. The spectrum of peptide 1\text{Met} at 150 µM in 10 mM potassium phosphate buffer at pH 7.4 shows a positive band at about 195 nm and a pronounced negative band at about 218 nm. The spectrum of peptide 1\text{Orn} shows only a negative band at about 200 nm. Figure 4.6 shows the circular dichroism spectra of peptides 1\text{Met} and 1\text{Orn}.

![Circular dichroism spectra of peptides 1\text{Met} and 1\text{Orn}](image.png)

**Figure 4.6.** Circular dichroism spectra of peptides 1\text{Met} and 1\text{Orn}. The spectra were acquired at 150 uM in 10 mM potassium phosphate buffer at pH 7.4.

Even though the sequences of peptides 1\text{Met} and 1\text{Orn} are nearly the same, the circular dichroism spectra are clearly not. The spectrum of peptide 1\text{Met} reflects that of a folded β-hairpin, but the spectrum of peptide 1\text{Orn} reflects that of an unstructured peptide. These spectra illustrate the influence of a single amino acid on β-sheet folding. Peptide 1\text{Met} contains the hydrophobic methionine residue, which is well-known to promote β-sheet folding.\textsuperscript{21,22} For peptide 1\text{Orn}, the hydrophilicity and charge of the ornithine residue appears to disrupt folding.
SDS-PAGE. SDS-PAGE is a common method to study mixtures of Aβ oligomers, because this method can separate different oligomers based on molecular weight. Here, I use SDS-PAGE to compare the oligomerization of peptides 1Met and 1Orn with trimer 2 (Figure 4.7). Tricine SDS-PAGE followed by silver staining shows that peptides 1Met and 1Orn migrate as single bands that are nearly identical, which appear at a molecular weight between 1.7 and 3.8 kDa. Trimer 2 migrates as two bands: a lower band at about 5.3 kDa and a higher band at about 10.6 kDa.

The molecular weights of the bands of peptides 1Met and 1Orn are consistent with species that are monomers or possibly dimers. The molecular weights of the trimer 2 bands are consistent with a monomer and dimer, respectively. These bands of trimer 2 migrate at molecular weights that are much larger than those of peptides 1Met and 1Orn, which further shows that peptides 1Met and 1Orn migrate as either monomers or dimers, but not trimers.

Figure 4.7. Silver-stained SDS-PAGE gel of peptide 1Met, peptide 1Orn, and trimer 4 in Tris buffer at pH 6.8 with 2% (w/v) SDS. Molecular weights calculated for the monomer, dimer, trimer, and hexamer are listed in parentheses.
Toxicity Studies of peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$. Evaluating the toxicity of Aβ oligomers toward neuronal cells is important for studying oligomerization and neurodegeneration relationships. Here, I evaluate the toxicity of peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$ toward the neuronally derived SH-SY5Y cells using a lactate dehydrogenase (LDH) release assay (Figure 4.8). The LDH assay showed that peptide $1_{\text{Met}}$ induces no LDH release at concentrations as high as 200 µM. In contrast, peptide $1_{\text{Orn}}$ increases LDH release in a dose-dependent fashion at 100, 150, and 200 µM.

![LDH release assay](image)

**Figure 4.8.** LDH release assay for peptides $1_{\text{Orn}}$ and $1_{\text{Met}}$ on SH-SY5Y cells (mean +/- SD, n=5).

Our laboratory has shown previously with peptides derived from Aβ and α-synuclein that oligomerization is important for toxicity.\textsuperscript{15,17,20} The LDH release assay of peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$ suggests that these two peptides differ in their solution-phase assembly, because $1_{\text{Orn}}$ is toxic but $1_{\text{Met}}$ is not. Although the lack of toxicity of peptide $1_{\text{Met}}$ may be the result of precipitation or fibril formation, precipitation was not observed in any of the wells in the 96-well plate. Since no difference in solution-phase assembly of peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$ was observed by SDS-PAGE, additional techniques are needed to further study oligomerization.
**1H NMR Spectroscopy.** I used 1H NMR spectroscopy to further evaluate whether oligomers of peptide 1Orn form in solution. The spectra show that peptide 1Orn oligomerizes in D2O with 25 mM CD3COOD and 25 mM CD3COONa at 500 MHz and 298 K (Figure 4.9). At concentrations less than 0.5 mM, the resonances of peptide 1Orn are sharp and distinct, indicating that the peptide is largely monomeric at these concentrations. At concentrations above 1 mM, the resonances are broadened due to oligomer formation. The oligomer resonances lack distinct features, particularly in the regions of the methyl protons (< 1.1 ppm), the α-protons (4.0 – 5.3 ppm), and the aromatic protons (6.5 – 7.4 ppm). The broad resonances are either due to exchange or the formation of a mixture of oligomers. The broad resonances also indicate that these oligomers do not form stable hydrogen bonds and hydrophobic contacts.
Figure 4.9. $^1$H NMR spectra of peptide $1_{Orn}$ at various concentrations in D$_2$O with 25 mM CD$_3$COOD and 25 mM CD$_3$COONa at 500 MHz and 298 K.
I prepared two analogues of peptide 1_{Orn} to interrogate the oligomerization of this peptide (Figure 4.10). I envisioned that incorporating polar residues in place of Val_{18} and Phe_{20} would disrupt the crystallographic hexamer, permitting the formation of a well-defined trimer and dodecamer. These residues would display on the minor surface and disrupt interactions between two trimers within a hexamer (Figure 4.10A). Peptide 3 is a single-mutant analogue that contains Tyr_{20} (Figure 4.10B). Peptide 4 is a double-mutant analogue that contains Thr_{18} and Tyr_{20} (Figure 4.10C).

**Figure 4.10.** X-ray crystallographic structure of the trimer formed by peptide 1_{Orn} (A, PDB 4NTR). Chemical structures of macrocyclic β-sheet peptides 3 and 4 (B and C).
The $^1$H NMR spectra of peptides 3 and 4 at 8 mM show broad resonances, but are less broad than that of peptide 1Orn. The broad resonances indicate that peptides 3 and 4 oligomerize in solution (Figure 4.11). These spectra also indicate that the polar Thr$_{18}$ and Tyr$_{20}$ residues facilitate the formation of oligomers that are more well defined, which suggests that peptides 3 and 4 are promising candidates for future studies by 2D $^1$H NMR spectroscopy.

**Figure 4.11.** $^1$H NMR spectra of peptides 1Orn, 3, and 4 at 8 mM in D$_2$O with 25 mM CD$_3$COOD and 25 mM CD$_3$COONa at 500 MHz and 298 K.

**Diffusion-Ordered Spectroscopy (DOSY).** DOSY measures the diffusion coefficient ($D$) of a molecule, which reflects the molecular weight.$^{23,24,25,26,27}$ The diffusion coefficient of an oligomer provides information on the size of the oligomer that forms.$^{28}$ Although SDS-PAGE and size-exclusion chromatography also provide information on size, these experiments can disrupt oligomers. The DOSY experiment does not. Here, I use DOSY to study the oligomers formed by peptides 1Orn, 3, and 4 and trimer 2 at various concentrations.

DOSY studies of peptide 1Orn at various concentrations show that an oligomer forms that is consistent with a dodecemer (Table 4.1). At 0.25 mM, peptide 1Orn gives a diffusion
The diffusion coefficient is associated with the monomer. The diffusion coefficient decreases to 13.2, 9.3, and \(8.6 \times 10^{-11}\) m\(^2\)/s upon increasing the concentration to 2.0, 4.0, and 16.0 mM. The steady decrease in diffusion coefficients indicates fast exchange between monomer and oligomer on the NMR time scale.\(^{29}\)

### Table 4.1. Diffusion coefficients \((D)\) in D\(_2\)O at 298 K

<table>
<thead>
<tr>
<th>MW(_{\text{monomer}}) ((\text{Da}))</th>
<th>MW(_{\text{trimer}}) ((\text{Da}))</th>
<th>conc. ((\text{mM}))</th>
<th>(D) ((10^{-11}) m(^2)/s)</th>
<th>oligomer state</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)(^\text{Orn})</td>
<td>1760</td>
<td>5280</td>
<td>0.25</td>
<td>20.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>18.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.0</td>
<td>13.2 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.0</td>
<td>9.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.0</td>
<td>9.1 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16.0</td>
<td>8.6 ± 1.3</td>
</tr>
<tr>
<td>(2)</td>
<td>5297(^b)</td>
<td></td>
<td>0.15</td>
<td>8.6 ± 1.7</td>
</tr>
<tr>
<td>(3)</td>
<td>1778</td>
<td></td>
<td>8.0</td>
<td>12.2 ± 0.5</td>
</tr>
<tr>
<td>(4)</td>
<td>1762</td>
<td></td>
<td>8.0</td>
<td>15.8 ± 0.9</td>
</tr>
</tbody>
</table>

\(^a\)Molecular weight calculated for the neutral (uncharged) peptide.  
\(^b\)Molecular weight calculated for the neutral (uncharged) cross-linked trimer.  
\(^c\)Oligomer state of the cross-linked trimer

The diffusion coefficient at 2.0 mM is about 0.65 times the monomer, which is consistent with the value of a trimer. The diffusion coefficients at 4.0, 6.0, and 8.0 mM are about 0.45 times that of the monomer, which indicates the formation of a higher-order oligomer that is much larger than a hexamer (0.55) but smaller than a decamer (0.46). The diffusion coefficient at 16.0 mM is about 0.42 times the monomer, which is comparable to the anticipated (0.44) ratio for a dodecamer.

I used DOSY NMR to study the oligomers formed by trimer \(2\) to confirm the size of the dodecamer formed by peptide \(1\)\(^\text{Orn}\). DOSY studies show that trimer \(2\) forms an oligomer consistent with a tetramer. The tetramer can also be thought of as a dodecamer of the monomer subunits. The DOSY spectrum of trimer \(2\) at 0.15 mM shows a single set of resonances, with a diffusion coefficient of about \(8.6 \times 10^{-11}\) m\(^2\)/s. This diffusion coefficient is comparable to the value of peptide \(1\)\(^\text{Orn}\) at 16.0 mM \((8.6 \times 10^{-11}\) m\(^2\)/s), indicating that the oligomers have a similar molecular weight.
DOSY studies show that peptides 3 and 4 have a lower propensity to oligomerize than peptide 1_{Orn}. At 8.0 mM, peptide 3 gives a diffusion coefficient of about $12.2 \times 10^{-11}$ m$^2$/s and peptide 4 gives a diffusion coefficient of about $15.8 \times 10^{-11}$ m$^2$/s. These diffusion coefficients are about 0.59 and 0.77 times that of a monomer, which are values consistent with a tetramer and dimer. These diffusion coefficients may also reflect larger oligomers in fast exchange with monomer, indicating lower association constants of peptides 3 and 4 than that of peptide 1_{Orn}.

To summarize the DOSY studies, the diffusion coefficients of peptides 1_{Orn}, 3, and 4, and trimer 2 show that these peptides oligomerize in solution. Peptide 1_{Orn} and trimer 2 form dodecamers and tetramers, which is intriguing since neither of these oligomers were observed by SDS-PAGE. The oligomerization of peptide 1_{Orn} may be important for the toxicity observed in the LDH assay. Even though the LDH assay was performed at micromolar concentrations of peptide 1_{Orn} and the dodecamer forms at millimolar concentrations, smaller oligomers may form within the cellular environment that induce toxicity.

**CONCLUSION**

Characterizing the structures, biophysical properties, and biological properties of Aβ oligomers is vital for gaining a better understanding of Alzheimer’s disease pathology. This chapter provides new information on the biophysical and biological properties of oligomers formed by peptides derived from Aβ$_{17-36}$. In this chapter, I evaluated the solution-phase assembly and biological properties of peptides 1_{Met} and 1_{Orn}, using $^1$H NMR spectroscopy, biophysical studies, and biological studies.

I used X-ray crystallography to establish that peptide 1_{Met} crystallizes in the same fashion as peptide 1_{Orn}, forming trimers, hexamers, and dodecamers in the crystal lattice. This finding is significant, because peptide 1_{Met} lacks the ornithine mutation and thus is a better mimic of Aβ.
The crystal structure also establishes that the trimers, hexamers, and dodecamers can tolerate both Met$_{35}$ and Orn$_{35}$ residues, which suggests that position 35 may even tolerate mutations to spectroscopic probes, fluorescent dyes, and other unnatural amino acids.

Although the structures of peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$ are nearly identical, their behavior in solution is different. SDS-PAGE studies showed that peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$ are largely monomeric. Circular dichroism showed that peptide $1_{\text{Met}}$ adopts a well-folded $\beta$-hairpin, while peptide $1_{\text{Orn}}$ is largely unstructured. An LDH assay shows that only peptide $1_{\text{Orn}}$ is toxic to cells and that peptide $1_{\text{Met}}$ is not. This assay suggests that peptide $1_{\text{Orn}}$ forms oligomers in the cell media that were not revealed by SDS-PAGE.

The $^1$H NMR studies of peptides $1_{\text{Met}}$, $1_{\text{Orn}}$, 3, and 4, and trimer 2 are the first major effort to characterize $N$-methylated macrocyclic $\beta$-sheet peptides by $^1$H NMR spectroscopy. The $^1$H NMR studies showed that peptide $1_{\text{Orn}}$ oligomerizes in solution, which provides an explanation for the toxicity observed in the LDH release assay. DOSY studies showed that the oligomers of peptide $1_{\text{Orn}}$ form dimers or trimers at low-millimolar concentrations, but appear to form a dodecamer at higher concentrations. DOSY studies also showed that trimer 2 oligomerizes to form a tetramer in solution, which has a comparable molecular weight to the dodecamer of peptide $1_{\text{Orn}}$. The dodecamer of peptide $1_{\text{Orn}}$ and the tetramer of trimer 2 were not observed previously by other methods. These NMR studies show that DOSY is a useful tool for evaluating the oligomers of $N$-methylated peptides. These NMR studies also provide a basis for using NMR to characterize oligomers formed by other $N$-methylated macrocyclic $\beta$-sheet peptides.
ACKNOWLEDGMENTS

I thank Dr. Huiying Li for the helpful advice and assistance, the Laser Spectroscopy Facility at the University of California, Irvine for assistance with the circular dichroism measurements, the National Institutes of Health (NIH) for funding (Grant GM097562), and the Stanford Synchrotron Radiation Lightsource (SSRL) and the Berkeley Center for Structural Biology (BCSB) of the Advanced Light Source (ALS) for synchrotron data collection. The Use of the Stanford Synchrotron Radiation Lightsource (SSRL) is jointly supported by the Department of Energy and the NIGMS. The BCSB is jointly supported by the NIH, NIGMS, and the Howard Hughes Medical Institute. The ALS is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.
REFERENCES AND NOTES


28. The ratio of oligomer and monomer diffusion coefficients reflects the oligomerization state of the oligomer. Dimers and trimers will have diffusion coefficients of about 0.75–0.79 and 0.66–0.69 times that of a monomer. Hexamers and dodecamers will have diffusion coefficients of about 0.55 and 0.44 times that of a monomer. A trimer can only marginally be distinguished from a trimer on the basis of diffusion coefficients measured by DOSY, but a hexamer could easily be distinguished from a dodecamer.

Efforts to Correlate Structure, Solution-Phase Behavior, and Toxicity of Peptides Derived from Aβ_{17–36}
4.I. MATERIALS AND METHODS

General

\(\text{N,N-Dimethylformamide (DMF), 2,4,6-collidine, N-methylmorpholine (NMM), and}
\) piperidine were purchased from Alfa Aesar and used without further purification. HPLC grade acetonitrile (CH\(_3\)CN) was purchased from Fisher Scientific and used without further purification. Methylene chloride (CH\(_2\)Cl\(_2\)) was purchased from Fisher Scientific, stored under argon, and passed through a column of alumina before use.\(^1\) Boc-Orn(Fmoc)-OH, HCTU, HBTU and HOBT were purchased from GL Biochem Ltd (Shanghai). 2-Chlorotrityl chloride resin and Fmoc protected amino acids were purchased from Chem-Impex International. \(\text{N,N-Diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), and triisopropylsilane (TIPS) were}
\) purchased from Oakwood Chemical. Deuterium oxide (D, 99.96%) was purchased from Cambridge Isotope Laboratories, Inc.

Synthesis of Peptides 1\text{Met}, 1\text{Orn}, 3, and 4

Synthesis and purification of peptides 1\text{Met}, 1\text{Orn}, 3, and 4, were performed as described previously.\(^2,3,4,5,6,7,8,9,10\)

Synthesis of Trimer 2

Synthesis, purification, and oxidation of trimer 2 were performed as described previously.\(^9\)
Crystallization of Peptide 1Met.

Initial crystallization conditions for peptide 1Met were based on the crystallization conditions previously published for peptide 1Orn, 0.1 M HEPES at pH 6.75 and 31% v/v Jeffamine M-600. These conditions were optimized for peptide 1Met using a 4x6 matrix Hampton VDX 24-well plate. In the optimized 24-well plate, the pH of HEPES in each row was varied by ± 0.1 pH units (6.3, 6.4, 6.5, and 6.6) and the Jeffamine concentration in each column was varied by ± 0.25% (24%, 24.25%, 24.5%, 24.75%, 25%, 25.25%, 25.5%). The first well in the 4x6 matrix (A1) was prepared by combining 100 μL of 1 M HEPES at pH 6.3, 480 μL of 50% (v/v) Jeffamine M-600 at pH 7.0, and 420 μL of 18 MΩ water to give a total volume of 1 mL. The conditions for the other wells were prepared in the same fashion by combining the appropriate amounts of 1 M HEPES buffer, 50% Jeffamine at pH 7.0, and 18 MΩ water to give a total volume of 1 mL.

Three hanging-drops were prepared per borosilicate glass slide by combining a solution of peptide 1Met (1 μL, 5 mg/mL) and the well solution (1 μL) in a ratio of 1:1, 2:1, and 1:2. Each slide was then inverted and placed on the corresponding well, using the silicone grease surrounding the well to create a hermetic seal. Large crystals grew after about 24 h. Crystals suitable for X-ray crystallography were harvested with a nylon loop attached to a copper pin and flash frozen in liquid nitrogen prior to data collection.
NMR Spectroscopy of Peptides

Sample Preparation. NMR spectroscopy of the macrocyclic \( \beta \)-sheet peptides was performed in D2O (D, 99.96%; Cambridge Isotope Laboratories, Inc.) with 25 mM CD3COOD (D, 99.5%; Cambridge Isotope Laboratories, Inc.) and 25 mM CD3COONa (D, 99%; Cambridge Isotope Laboratories, Inc.). Each solution contained 0.06 mM 4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA) as an internal standard for referencing chemical shifts. The solutions were prepared by dissolving a weighed portion of the peptide in the appropriate volume of solvent. The molecular weights of the peptides were calculated as the TFA salts with all amino groups assumed to be protonated. The solutions were allowed to stand for at least 1 h to allow complete hydrogen to deuterium exchange of the amide NH protons.

\(^1\text{H NMR, TOCSY and ROESY Data Collection.}\) NMR spectra were recorded on a Bruker 500 MHz spectrometer with a TCI probe. Presaturation water suppression was applied as needed. TOCSY spectra were recorded with 2048 points in the \( f_2 \) dimension and 512 increments in the \( f_1 \) dimension with a 150-ms spin-lock mixing time. ROESY spectra were recorded with 2048 points in the \( f_2 \) dimension and 512 increments in the \( f_1 \) dimension with a 200-ms spin-lock mixing time.

\(^1\text{H NMR, TOCSY and ROESY Data Processing.}\) NMR spectra were processed with Bruker XwinNMR software. Automatic baseline correction was applied in both dimensions after phasing the spectra. TOCSY and ROESY spectra were Fourier transformed to a final matrix size of 2048 x 1024 real points using a Qsine weighting function and forward linear prediction. NOESY spectra were Fourier transformed to a final matrix size of 2048 x 2048 real points using a Qsine weighting function and forward linear prediction.
**Diffusion-Ordered Spectroscopy (DOSY) Experiments.** DOSY experiments were performed on a Bruker 500 MHz spectrometer equipped with a TCI cryoprobe, with a diffusion delay (Δ) of 75-ms and a diffusion gradient length (δ) of 2.5-ms. Sixteen sets of FIDs were recorded with the gradient strength incremented from 5%–95% using a linear ramp. The combined FIDs were Fourier transformed in Bruker's TopSpin™ software to give a pseudo-2D spectrum. After phasing and performing baseline correction, each pseudo-2D spectrum was processed with logarithmic scaling on the Y-axis. The Y-axis was calibrated to the diffusion coefficient of the residual HOD peak in D₂O (1.9 x 10⁻⁹ m²/s at 298 K). The diffusion coefficients of the peptides were read and converted from logarithmic values to linear values.

**SDS-PAGE of Peptides 1Met, 1Orn, and Trimer 2.**

Reagents and gels for SDS-PAGE were prepared according to recipes and procedures for Tricine–SDS-PAGE as described by Schägger, H. in *Nat. Protoc.* 2006, 1, 16–22.¹¹ The migration of the peptides was compared with a molecular weight protein ladder (SpectraTM Multicolor Low Range Protein Ladder, ThermoFisher Scientific, catalog #: 26628). The peptides in the gel were visualized by staining them with silver nitrate, which was performed using the procedures and reagents described by Simpson, R. J. *CSH Protoc.*, 2007.¹² Fresh solutions were prepared each time of the 0.02% (w/v) sodium thiosulfate, 0.1% (w/v) silver nitrate solution, and developing solution.

*Sample preparation.* SDS-PAGE of peptides 1Met, 1Orn, and trimer 2 was performed with aliquots from 10 mg/mL stock solutions. The solutions were diluted with 18 MΩ water to create
2-mg/mL solutions of peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$ and a 0.12-mg/mL solution of trimer 2. The 2-mg/mL and 0.12-mg/mL solutions were further diluted with a 2X SDS-PAGE loading buffer (100 mM Tris buffer at pH 6.8, 20% (v/v) glycerol, and 4% SDS) to a final concentration of 1 mg/mL for peptides $1_{\text{Met}}$ and $1_{\text{Orn}}$, and 0.06 mg/mL for trimer 2. 5.0 μL of each solution was added into the lanes of a 4% polyacrylamide stacking gel with a 16% polyacrylamide running gel. 2.0 μL of the protein ladder was added into an adjacent lane. The gel was run at a constant 90 volts.

**Gel Staining.** After electrophoresis, the gel was removed from the casting glass and agitated gently in fixing solution [50% (v/v) methanol and 5% (v/v) acetic acid in deionized water] for 20 min. The fixing solution was then discarded and the gel was agitated gently in 50% (v/v) aqueous methanol for 10 min. The 50% methanol was discarded and the gel was agitated gently in deionized water for 10 min. The water was discarded and the gel was agitated gently in 0.02% (w/v) sodium thiosulfate in 18 MΩ water for 1 min. The sodium thiosulfate was discarded and the gel was rinsed twice with deionized water for 1 min. After the last rinse, the gel was submerged in chilled 0.1% (w/v) silver nitrate in 18 MΩ water and rocked at 4 °C for 20 min. The silver nitrate solution was then discarded and the gel was rinsed twice with 18 MΩ water for 1 min. To develop the gel, the gel was incubated in developing solution (2% (w/v) sodium carbonate, 0.04% (w/v) formaldehyde until the desired intensity of staining was reached (ca. 1–3 min). The development was then stopped by discarding the developing solution and submerging the gel in 5% aqueous acetic acid.
LDH release assays.

The LDH release assay was performed using the Pierce LDH Cytotoxicity Assay Kit from Thermo Scientific. Experiments were performed in replicates of five, and an additional 10 wells were used for controls. Cells were cultured in the inner 60 wells (rows B–G, columns 2–11) of the 96-well plate. DMEM:F12 media (100 μL) was added to the outer wells (rows A and H and columns 1 and 12), in order to ensure the greatest reproducibility of data generated from the inner wells.

Sample Preparation. Preparation of stock solutions of peptides $\text{1Met}$ and $\text{1Orn}$. 10-mg/mL stock solutions of peptides $\text{1Met}$ and $\text{1Orn}$ were prepared gravimetrically by dissolving 1.0 mg of each compound in 100 μL of deionized water that was filtered through a 0.2 μm syringe filter. The stock solution was used to create 2-, 1.5, 1.0, and 0.5 mM working solutions of peptides $\text{1Met}$ and $\text{1Orn}$.

Tissue Culture. Preparation of SH-SY5Y cells for LDH release assays. SH-SY5Y cells were plated in a 96-well plate at 15,000 cells per well. Cells were incubated in 100 μL of a 1:1 mixture of DMEM:F12 media supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO$_2$ atmosphere and allowed to adhere to the bottom of the plate for 24 hours.

Cell Treatment. After 24 hours, the culture media was removed and replaced with 90 μL of serum-free DMEM:F12 media. A 10-μL aliquot of the working solution of peptides $\text{1Met}$ and $\text{1Orn}$ was added to each well, for well concentrations of 200, 150, 100, and 50 μM. Experiments were run in replicates of five. Five wells were used as controls and received 10-μL aliquots of
deionized water (vehicle). Another five wells were left untreated, to be subsequently used as controls with lysis buffer for the LDH release assay. Cells were incubated at 37 °C in a 5% CO2 atmosphere for 72 hours.

**LDH release assay.** After 72 hours, 10 μL of 10x lysis buffer—including with the assay kit—was added to the five untreated wells, and the cells were incubated for an additional 45 min. After 45 min, a 50-μL aliquot of the supernatant media from each well was transferred to a new 96-well plate and 50 μL of LDH substrate solution, prepared according to manufacturer’s protocol, was added to each well. The treated plates were stored in the dark for 30 min.

**Measuring Cell Death.** The absorbance of each well was measured at 490 and 680 nm (A490 and A680). Data were processed by calculating the differential absorbance for each well (A490−A680) and comparing those values to those of the lysis buffer controls and the untreated controls:

\[
\% \text{ cell death} = \frac{[\text{A490−A680} \text{compound} − \text{A490−A680} \text{vehicle}]}{[\text{A490−A680} \text{lysis} − \text{A490−A680} \text{vehicle}]}\]
4.II. REFERENCES


235
4.III. CHARACTERIZATION DATA
RP-HPLC of peptide $^{1}_{\text{Met}}$

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Totals: 3232.95288 531.77228

MS (ESI) of peptide $^{1}_{\text{Met}}$

Calculated $m/z$ for $^{85}\text{C}_{136}\text{N}_{18}\text{O}_{20}\text{S}_{1}$:

- $[\text{M+H}]^+$: 1762.00
- $[\text{M+2H}]^{2+}$: 881.50

column: Aeris XB-C18 2.6µ
dimensions: 150 mm x 4.6 mm
mobile phase: A: H$_2$O, 0.1% TFA
B: CH$_3$CN, 0.1% TFA
gradient: A/B (95:5) to (0:67) in 15 min
flow rate: 1.0 mL/min
detection: VWD, wavelength = 214 nm
temperature: 298 K
MS (ESI) of peptide 1Met

\[ [\text{M+H}]^+ \]

\[ [\text{M+Na}]^+ \]

\[ [\text{M+2H}]^{2+} \]

NT_V_171024_1Met_1-1 10 (0.184) Sm (SG, 2x3.00); Cm (8:12) TOF MS ES+

1.17e3
$^1$H NMR of peptide 1Met, *0.5 mM in D$_2$O with 50 mM acetate buffer at 500 MHz and 293 K
Precipitation was observed in the NMR tube, resulting in a lower sample concentration.
The asterisk (*) indicates that the sample concentration is only nominally 0.5 mM.
RP-HPLC of peptide 1Orn

Column: Aeris XB-C18 2.6µ
Dimensions: 150 mm x 4.6 mm
Mobile phase: A: H2O, 0.1% TFA
B: CH3CN, 0.1% TFA
Gradient: A/B (95:5) to (0:67) in 15 min
Flow rate: 1.0 mL/min
Detection: VWD, wavelength = 214 nm
Temperature: 298 K

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Totals: 1998.96826 460.10202

MS (ESI) of peptide 1Orn

NT_V_171024_1Orn_2-1_11 (0.202) Sm (SG, 2x3.00); Cm (9:18)

Calculated m/z for C85H137N19O20:

[M+H]+: 1745.04
[M+2H]2+: 873.02
$^1$H NMR of peptide 1Orn, 0.25 mM in D$_2$O with 50 mM acetate buffer at 500 MHz and 293 K
$^1$H NMR of peptide 1Orn, 0.25 mM in D$_2$O with 50 mM acetate buffer at 500 MHz and 293 K
$^1$H NMR of peptide 1Orn, 0.25 mM in D$_2$O with 50 mM acetate buffer at 500 MHz and 293 K
$^1$H NMR of peptide 1Orn, 0.25 mM in D$_2$O with 50 mM acetate buffer at 500 MHz and 293 K
$^1$H NMR of peptide 1Orn, 0.25 mM in D$_2$O with 50 mM acetate buffer at 500 MHz and 293 K
$^1$H NMR 2D TOCSY of peptide 1Orn with presaturation suppression of the HOD peak 0.25 mM in D$_2$O at 500 MHz and 298 K with 150-ms spin-lock mixing time
\(^1\)H NMR 2D TOCSY of peptide Orn with presaturation suppression of the HOD peak 0.25 mM in D\(_2\)O at 500 MHz and 298 K with 150-ms spin-lock mixing time
$^1$H NMR 2D ROESY of peptide 1Orn with presaturation suppression of the HOD peak 0.25 mM in D$_2$O at 500 MHz and 298 K with 150-ms spin-lock mixing time
$^1$H NMR 2D ROESY of peptide 1Orn with presaturation suppression of the HOD peak 0.25 mM in D$_2$O at 500 MHz and 298 K with 150-ms spin-lock mixing time
$^{1}$H NMR DOSY of macrocyclic β-sheet peptide 1Orn at 500 MHz and 298 K
0.25 mM in 50 mM deuterioacetate buffer in D$_2$O

Calculations for macrocyclic β-sheet peptide 1Orn at 0.25 mM

$D_{HOD} = 19.0 \times 10^{-10}$ m$^2$/s $^a$

$\log (D_{HOD}) = -8.721$

$D_{\text{monomer}} : \log(D) = -9.69; D = 10^{-9.69} = 20.4 \pm 1.0 \times 10^{-11}$ m$^2$/s

$^1$H NMR DOSY of macrocyclic β-sheet peptide $\text{10}_{\text{orn}}$ at 500 MHz and 298 K
0.5 mM in 50 mM deuterioacetate buffer in D$_2$O

Calculations for macrocyclic β-sheet peptide $\text{10}_{\text{orn}}$ at 0.5 mM

$D_{\text{HOD}} = 19.0 \times 10^{-10}$ m$^2$/s $^a$

$\log(D_{\text{HOD}}) = -8.721$

$D_{\text{oligomer}}: \log(D) = -9.74; D = 10^{-9.74} = 18.2 \pm 1.2 \times 10^{-11}$ m$^2$/s

$^1$H NMR DOSY of macrocyclic $\beta$-sheet peptide $\mathbf{1}_{\text{Oro}}$ at 500 MHz and 298 K
2 mM in 50 mM deuterioacetate buffer in D$_2$O

Calculations for macrocyclic $\beta$-sheet peptide $\mathbf{1}_{\text{Oro}}$ at 2 mM

$D_{\text{HOD}} = 19.0 \times 10^{-10}$ m$^2$/s $^a$

$\log (D_{\text{HOD}}) = -8.721$

$D_{\text{oligomer}}: \log(D) = -9.88; D = 10^{-9.88} = 13.2 \pm 1.0 \times 10^{-11}$ m$^2$/s

$^1$H NMR DOSY of macrocyclic $\beta$-sheet peptide $1_{\text{Orn}}$ at 500 MHz and 298 K
4 mM in 50 mM deuterioacetate buffer in D$_2$O

Calculations for macrocyclic $\beta$-sheet peptide $1_{\text{Orn}}$ at 4 mM

$D_{\text{HOD}} = 19.0 \times 10^{-10}$ m$^2$/s $^a$

$\log (D_{\text{HOD}}) = -8.721$

$D_{\text{oligomer}}: \log(D) = -10.03; D = 10^{-10.03} = 9.3 \pm 1.1 \times 10^{-11}$ m$^2$/s

$^{1}H$ NMR DOSY of macrocyclic β-sheet peptide $1_{\text{Orn}}$ at 500 MHz and 298 K
6 mM in 50 mM deuterioacetate buffer in D$_2$O

Calculations for macrocyclic β-sheet peptide $1_{\text{Orn}}$ at 6 mM

$D_{\text{HOD}} = 19.0 \times 10^{-10}$ m$^2$/s

$\log (D_{\text{HOD}}) = -8.721$

$D_{\text{oligomer}} : \log(D) = -10.04 ; D = 10^{-10.04} = 9.1 \pm 0.9 \times 10^{-11}$ m$^2$/s

$^1$H NMR DOSY of macrocyclic β-sheet peptide 1Orn at 500 MHz and 298 K
8 mM in 50 mM deuterioacetate buffer in D$_2$O

Calculations for macrocyclic β-sheet peptide 1Orn at 8 mM

$D_{HOD} = 19.0 \times 10^{-10}$ m$^2$/s $^a$

$\log (D_{HOD}) = -8.721$

$D_{oligomer}: \log (D) = -10.04; D = 10^{-10.04} = 9.1 \pm 1.0 \times 10^{-11}$ m$^2$/s

$^1$H NMR DOSY of macrocyclic $\beta$-sheet peptide $1_{\text{Orn}}$ at 500 MHz and 298 K
16 mM in 50 mM deuterioacetate buffer in D$_2$O

Calculations for macrocyclic $\beta$-sheet peptide $1_{\text{Orn}}$ at 16 mM

$D_{\text{HOD}} = 19.0 \times 10^{-10}$ m$^2$/s \textit{a}

$\log (D_{\text{HOD}}) = -8.721$

$D_{\text{oligomer}}: \log(D) = -10.068; \quad D = 10^{-10.068} = 9.1 \pm 1.3 \times 10^{-11}$ m$^2$/s

$^1$H NMR of trimer 2, 0.15 mM in D$_2$O with 50 mM acetate buffer at 500 MHz and 293 K
$^1$H NMR DOSY of macrocyclic $\beta$-sheet trimer 2 at 500 MHz and 298 K
0.15 mM in 50 mM deuterioacetate buffer in D$_2$O

Calculations for macrocyclic $\beta$-sheet trimer 2 at 0.15 mM

$D_{HOD} = 19.0 \times 10^{-10}$ m$^2$/s $^a$

$\log (D_{HOD}) = -8.721$

$D_{oligomer} : \log(D) = -10.065; D = 10^{-10.065} = 8.6 \pm 1.7 \times 10^{-11}$ m$^2$/s

$^1$H NMR DOSY of macrocyclic $\beta$-sheet peptide 3 at 500 MHz and 298 K
8 mM in 50 mM deuterioacetate buffer in D$_2$O

Calculations for macrocyclic $\beta$-sheet peptide 3 at 8 mM

$D_{HOD} = 19.0 \times 10^{-10}$ m$^2$/s

log $(D_{HOD}) = -8.721$

$D_{oligomer}$: log$(D) = -9.914$; $D = 10^{-9.914} = 12.2 \pm 0.5 \times 10^{-11}$ m$^2$/s

$^1$H NMR DOSY of macrocyclic $\beta$-sheet peptide 4 at 500 MHz and 298 K
8 mM in 50 mM deuterioacetate buffer in D$_2$O

Calculations for macrocyclic $\beta$-sheet peptide 4 at 8 mM

$D_{HOD} = 19.0 \times 10^{-10}$ m$^2$/s $^a$

log ($D_{HOD}$) = -8.721

$D_{oligomer}$ : log($D$) = -9.8; $D = 10^{-9.8} = 15.8 \pm 0.9 \times 10^{-11}$ m$^2$/s